ANNUAL REPORTS IN MEDICINAL CHEMISTRY
Volume 30

Sponsored by the Division of Medicinal Chemistry of the American Chemical Society

EDITOR-IN-CHIEF:

JAMES A. BRISTOL

PARKE-DAVIS PHARMACEUTICAL RESEARCH
DIVISION OF WARNER-LAMBERT COMPANY
ANN ARBOR, MICHIGAN

SECTION EDITORS

ANNETTE M. DOHERTY • WILLIAM K. HAGMANN • JOHN C. LEE
JACOB J. PLATTNER • DAVID W. ROBERTSON • MICHAEL C. VENUTI

EDITORIAL ASSISTANT

LISA BAUSCH

ACADEMIC PRESS

San Diego New York Boston London Sydney Tokyo Toronto

This book is printed on acid-free paper. ♾

Copyright © 1995 by ACADEMIC PRESS, INC.

All Rights Reserved.
No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or any information storage and retrieval system, without permission in writing from the publisher.

Academic Press, Inc.
A Division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495

United Kingdom Edition published by
Academic Press Limited
24-28 Oval Road, London NW1 7DX

International Standard Serial Number: 0065-7743

International Standard Book Number: 0-12-040530-X

PRINTED IN THE UNITED STATES OF AMERICA
95 96 97 98 99 00 MM 9 8 7 6 5 4 3 2 1

CONTRIBUTORS

Artis, Dean R.	265	Liaw, Chen W.	21
Barrett, John F.	111	Lodge, David	51
Behan, Dominic P.	21	Lovenberg, Timothy W.	21
Beutler, Bruce	239	Matthews, David J.	275
Bloom, Jonathan D.	189	McCarthy, James R.	21
Boehme, Richard E.	139	McDonald, Ian A.	41
Borthwick, Alan D.	139	McDowell, Robert S.	265
Botfield, Martyn C.	227	McSwiggen, James A.	285
Bowman, David	51	Mitscher, Lester A.	329
Chalmers, Derek T.	21	Nadzan, Alex M.	119
Cheng, Xue-Min	295	Nagy, Zoltan A.	199
Claus, Thomas H.	189	Neote, Kuldeep	209
Cooper, Kelvin	209	Post, Leonard E.	219
Cosford, Nicholas	41	Puttfarcken, Pamela	31
De Souza, Errol B.	21	Raffa, Robert B.	11
Dell, Colin	51	Rasmussen, Kurt	1
Denner, Larry A.	61	Reitz, Allen B.	11
Dirlam, John P.	101	Reitz, David B.	179
Dixon, Richard A.F.	61	Rocco, Vincent P.	1
Dow, Robert L.	159	Russell, Keith	81
Empfield, James R.	81	Scanlan, Thomas S.	255
Ganellin, C. Robin	329	Scarborough, Robert M.	71
Gilmore, Jeremy	51	Seibert, Karen	179
Gishizky, Mikhail L.	247	Shiosaki, Kazumi	31
Goldman, Robert C.	129	Stirling, David	319
Goulet, Mark T.	169	Tanaka, S. Ken	151
Green, Jeremy	227	Thompson, W. Leigh	339
Grigoriadis, Dimitri E.	21	Topliss, John G.	329
Isaacson, Richard E.	111	Usman, Nassim	285
Jaynes, Burton H.	101	Vernier, Jean-Michel	41
Jefson, Martin R.	101	Vidovic´, Damir	199
Jetter, Michele C.	11	Walsh, Thomas F.	91
Klein, Larry L.	151	Wild, Kenneth D.	11
Kreutter, David K.	159	Wyatt, Paul G.	139

PREFACE

Annual Reports in Medicinal Chemistry continues to strive to provide timely and critical reviews of important topics in medicinal chemistry together with an emphasis on emerging topics in the biological sciences which are expected to provide the basis for entirely new future therapies.

Volume 30 retains the familiar format of previous volumes, this year with 34 chapters. Sections I - IV are disease oriented and generally report on specific medicinal agents with updates from Volume 29 on anti-infectives, antivirals, and endothelin. We have continued the trend of reducing the number of annual updates in favor of specifically focussed and mechanistically oriented chapters, where the objective is to provide the reader with the most important new results in a particular field. To this end, chapters on topics not reported in at least two years include: serotonin, analgesics, CRF, neuroinflammation, nicotinic acetylcholine receptors, neuronal calcium channels, *vascular proliferative diseases, thrombin, potassium channels, retinoids,* β-D glucans, *Helicobacter pylori,* diabetes, GnRH, COX-2, β_3-agonists, T-cell antigen, and chemokines.

Sections V and VI continue to emphasize important topics in medicinal chemistry, biology, and drug design as well as the important interfaces among these disciplines. Included in Section V, Topics in Biology, are chapters on gene therapy, Src homology domains, apoptosis, and protein tyrosine kinase. Some of the topics of research reviewed in this section will appear in a chapter related to medicinal agents in a future volume, once sufficient time has passed to allow new compounds to be developed from a biological strategy. Chapters in Section VI, Topics in Drug Design and Discovery, reflect the current focus on mechanism-directed drug discovery and newer technologies. These include chapters on catalytic antibodies, structure-based *design, biosensor technology, and ribozymes.*

Volume 30 concludes with Trends and Perspectives, with chapters on NCE introductions worldwide in 1994, thalidomide in AIDS, the results of a survey on educating medicinal chemists, and a perspective on investing in pharmaceutical research in the era of managed care. In addition to the chapter reviews, a comprehensive set of indices has been included to enable the reader to easily locate topics in volumes 1-30 of this series.

Over the past year, it has been my pleasure to work with 6 highly professional section editors and 68 authors, whose critical contributions comprise this volume.

James A. Bristol
Ann Arbor, Michigan
May, 1995

SECTION I. CENTRAL NERVOUS SYSTEM DISEASES

Editor: David W. Robertson, Ligand Pharmaceuticals
San Diego, CA 92121

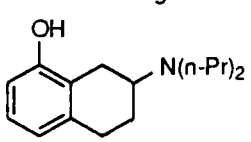
Chapter 1. Recent Progress in Serotonin (5-HT)$_{1A}$ Receptor Modulators

Kurt Rasmussen and Vincent P. Rocco
Central Nervous System Research
Eli Lilly & Co., Indianapolis, IN 46285

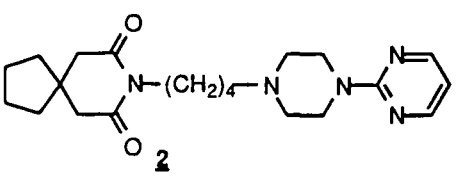
Introduction - This chapter will begin with a brief introduction to serotonin (5-hydroxytryptamine; 5-HT) and the 5-HT$_{1A}$ receptor subtype. The introduction will be followed by a discussion of recent developments in the potential clinical utility of selective 5-HT$_{1A}$ agonists and antagonists in anxiety, Alzheimer's disease, emesis, depression, and prostate cancer. The chapter will conclude with a description of the most recently synthesized selective 5-HT$_{1A}$ ligands.

5-HT is a biogenic indolamine found in the blood, gastro-intestinal tract, and central nervous system. In the blood, 5-HT plays a role in vascular tone and platelet function, while in the gastro-intestinal system 5-HT has strong effects on intestinal motility and gastric-acid secretion. In the brain, 5-HT is synthesized and released as a neurotransmitter from specific cells that form a discrete brain system. The cell bodies of this brain system are localized almost exclusively along the midline of the brainstem within the raphe nuclei; however these neurons have extensive projections throughout the neuraxis and innervate virtually every major part of the central nervous system from the cerebral cortex to the spinal cord (1). 5-HT has been hypothesized to play a role in a myriad number of physiological processes, behaviors, and disease states (1-4). The many actions of 5-HT in the body are mediated through at least fourteen different receptor subtypes (5-8). All of these subtypes have been cloned since 1987, with most of them cloned since 1992. Except for the 5-HT$_3$ receptor, which is a ligand-gated ion channel, all 5-HT receptor subtypes so far discovered interact with G proteins. One of these receptor subtypes, the 5-HT$_{1A}$ receptor, is found in high concentrations in the limbic system (e.g., hippocampus, septum, entorhinal cortex, and amygdala) where it is thought to play a role in emotional processes (5).

8-OH-DPAT (1), buspirone (2), and flesinoxan (3) are selective agonists or partial agonists for the 5-HT$_{1A}$ receptor (9). Activation of the 5-HT$_{1A}$ receptor leads to a number of physiological changes that can be easily quantified. For example, administration of 5-HT$_{1A}$ agonists can result in a temperature decrease (10), an increase in serum corticosterone (11), an inhibition of firing of 5-HT-containing neurons (12), and a characteristic set of motor changes collectively known as the serotonin motor syndrome (13). These changes in response to 5-HT$_{1A}$ agonists provide several easily quantifiable *in vivo* parameters with which to measure the effectiveness of 5-HT$_{1A}$ agonists and antagonists.

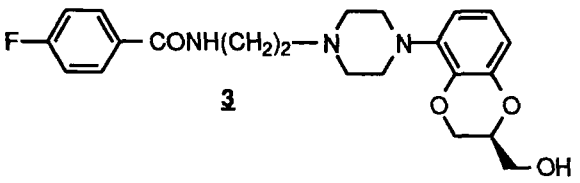


1



2

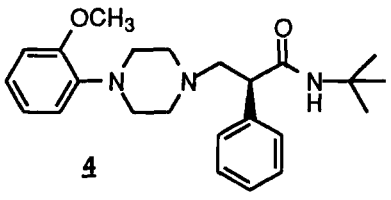
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.



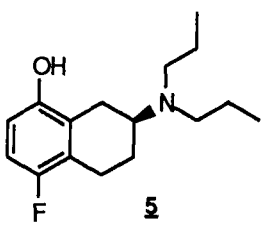
3

POTENTIAL CLINICAL APPLICATIONS

Anxiety - 5-HT has been hypothesized to play a role in anxiety (14,15). This hypothesis has been supported, in part, by the clinical efficacy of compounds that alter serotonergic transmission. For example, the 5-HT_{1A} partial agonist buspirone has anxiolytic effects in generalized anxiety disorder and 5-HT reuptake blockers are effective treatments for panic disorder (16,17). Recently, the 5-HT_{1A} full agonists flesinoxan and S 20499 (*vide infra*) have been reported to display anxiolytic-like activity in several animal models of anxiety (18-20). Indeed, preliminary clinical trials indicate that flesinoxan is effective in the treatment of generalized anxiety disorder, but not panic disorder, in man (21,22). Interestingly, albeit no clinical reports have been published, 5-HT_{1A} antagonists also have been shown to have anxiolytic activity in several animal models. For example, WAY 100135 and its (+)-enantiomer (4) have displayed anxiolytic-like activity in the elevated plus-maze and light/dark box models in mice (23,24). In addition, the 5-HT_{1A} antagonist (*S*)-UH-301 (5) has anxiolytic-like activity in the elevated plus-maze in rats and the light/dark box in mice, but not in the conflict test in rats (25). 5-HT_{1A} antagonists will be discussed in detail in a subsequent section of this chapter.



4



5

As is seen with buspirone, flesinoxan requires several weeks of administration to achieve its full anxiolytic effect in man. A novel hypothesis concerning morphological changes in the brain has recently been proposed to explain this delayed onset (26). Activation of 5-HT_{1A} receptors located on astroglial cells in the brain leads to the release of a neurotrophic agent S-100ß (27). This factor can then cause regeneration or sprouting of neuronal terminals which may have been lost due to the disease process. Thus, the delayed onset of efficacy for 5-HT_{1A} agonists may result from the length of time required to fully achieve the necessary physical changes in the morphology of neurons.

Alzheimer's Disease - Recently 5-HT_{1A} antagonists have also been hypothesized to be potentially useful for the treatment of Alzheimer's disease. The degeneration of cortical pyramidal neurons may lead to glutamatergic hypoactivity and cognitive impairment in Alzheimer's disease (28). Similar types of neurons in the rat appear to contain 5-HT_{1A} receptors (29), and activation of 5-HT_{1A} receptors can hyperpolarize a subset of human cortical pyramidal neurons (30). Therefore, in the brain of an Alzheimer's patient, a 5-HT_{1A} antagonist could prevent the hyperpolarizing effects of 5-HT on the proportion of pyramidal neurons not yet degenerated, thus relieving the glutamatergic hypoactivity (31). This hypothesis remains to be tested clinically.

Nausea and vomiting - While 5-HT$_3$ antagonists are currently marketed for the relief of postoperative and chemotherapy-induced nausea, 5-HT$_{1A}$ agonists have also shown preclinical efficacy in this regard. In fact, animal studies have shown that 5-HT$_{1A}$ agonists are more of a broad-spectrum antiemetic since they are effective in motion sickness, xylazine, and cisplatin-induced emesis, while 5-HT$_3$ antagonists are effective only against cisplatin-induced emesis (32). Recently, flesinoxan was shown to be more potent than 8-OH-DPAT in suppressing motion-sickness and xylazine-induced emesis in cats (33). The finding that flesinoxan is more potent than 8-OH-DPAT in these assays is surprising given that most *in vivo* studies find the opposite. In addition, in these studies the 5-HT$_{1A}$ antagonist (-)propranolol suppresses the effects of 8-OH-DPAT but not of flesinoxan, while the 5-HT$_{1A}$ weak partial agonist NAN-190 blocks the effects of higher doses of flesinoxan but does not block the effects of 8-OH-DPAT. Subtypes of the 5-HT$_{1A}$ receptor may account for these discrepant findings.

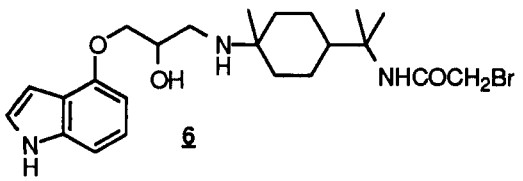
Depression - Selective 5-HT reuptake inhibitors (SSRIs) will inhibit the uptake of 5-HT and increase the amount of synaptic 5-HT soon after administration (37). However, weeks of treatment with SSRIs is required to achieve antidepressant effects in man. These ostensibly discrepant observations may be explained by the finding that 5-HT neurons have 5-HT$_{1A}$ receptors on their dendrites and cell bodies (i.e., autoreceptors; these receptors are post-synaptic to projections from other 5-HT cells), and activation of these autoreceptors leads to an inhibition of cell firing. Thus, acute administration of an SSRI increases the synaptic levels of 5-HT but also decreases the activity of 5-HT neurons. This inhibition of cell body activity may be thought of as a "brake" on the system which limits the amount of extracellular 5-HT obtained after administration of an SSRI. Recent studies have shown that this inhibitory effect of SSRIs on 5-HT neuronal firing is decreased upon chronic administration (38,39). This decreased inhibition of firing is thought to be achieved through a desensitization of the 5-HT$_{1A}$ autoreceptor. Thus, the desensitization of 5-HT$_{1A}$ autoreceptors has been hypothesized to account for the delayed onset of antidepressant action in the clinic (40).

Experiments published in the last year have both questioned and supported the hypothesis that 5-HT$_{1A}$ autoreceptors become desensitized upon chronic administration of an SSRI (41-44). While it remains to be determined whether autoreceptor desensitization can fully explain the delayed onset of clinical response to SSRIs, the stimulation of 5-HT$_{1A}$ autoreceptors does appear to limit the levels of extracellular 5-HT obtained after administration of an SSRI. Recent publications have shown that pharmacological blockade of the 5-HT$_{1A}$ receptor with an antagonist will increase the amount of extracellular 5-HT seen following the administration of an SSRI (45,46). Thus, the addition of a 5-HT$_{1A}$ antagonist to treatment with an SSRI could prevent the inhibition of activity of 5-HT neurons, leading to increased 5-HT release, and ultimately to a faster and better antidepressant action. Indeed, in an open-label trial the addition of pindolol (which has 5-HT$_{1A}$ antagonists properties) induced a dramatic and rapid improvement of the antidepressant efficacy of SSRIs and monoamine oxidase (MAO) inhibitors (47). In these studies, the onset of action was reduced and a rapid and full antidepressant response was seen in patients previously resistant to treatment with SSRIs or MAO inhibitors. A second open-label trial reported similar effects of pindolol on the onset of action of the SSRI paroxetine in untreated depressed patients and on the antidepressant effect of paroxetine, fluoxetine and moclobemide in drug-resistant depressed patients (48). Interestingly, the addition of pindolol did not lead to an antidepressant response in drug-resistant patients receiving the SSRI sertraline. The reason for the lack of enhancement of sertraline by pindolol is unclear. While these studies are exciting they do have limitations. First, to control for placebo effects, the findings must be replicated in double-blind studies. Second,

since pindolol has a higher affinity for the beta-adrenergic receptor than the 5-HT_{1A} receptor, studies with more potent and selective 5-HT_{1A} antagonists would help to delineate the exact mechanism of action of pindolol.

In preliminary clinical trials, the 5-HT_{1A} full agonist flesinoxan has also been reported have antidepressant actions (49,50). However, several weeks of treatment is also needed to see efficacy. This delayed onset could be mediated by the same down-regulation of 5-HT_{1A} receptors hypothesized to play a role in the antidepressant action of SSRIs.

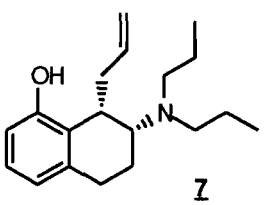
Prostate cancer - In addition to a role as a neurotransmitter, 5-HT can also function as a growth factor. 5-HT is found in most neurendocrine cells of the human prostate and in human prostate tumor cell lines where it may play a role in the progression of prostate carcinoma (34,35). Recently, the 5-HT_{1A} antagonist pindobind (**6**) has been shown to inhibit the growth of the human prostate tumor cell lines PC3, DU-145, and LNCaP *in vitro* (36). In addition, in athymic nude mice, pindobind had marked growth-inhibitory effects on the aggressive PC-3 cell line (36). Thus, while additional compounds need to be studied, 5-HT_{1A} antagonists could be a useful treatment for prostate cancer.



6

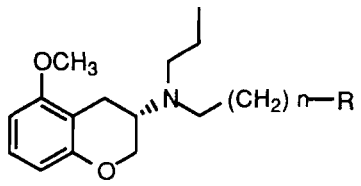
SELECTIVE 5-HT_{1A} AGONISTS

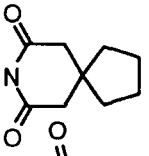
Research into selective 5-HT_{1A} receptor agonists continues (51). Several new 5-HT_{1A} agonists based upon previously known members of these structural classes have been reported. A structure-activity relationship (SAR) study of the racemic, C-1 and C-3 substituted 8-OH-DPAT analogues, **7**, demonstrated that highest 5-HT_{1A} agonist activity is obtained for the *cis*, C-1 substituted derivatives, with allyl being the optimal substituent, while *cis* C-3 substitution abrogates activity completely (52).

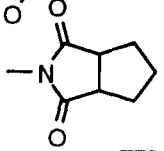


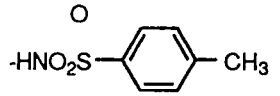
7

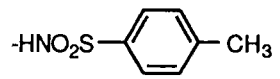
Benzopyran derivatives (**8**-**11**), were shown to be full agonists of the 5-HT_{1A} receptor, (IC_{50} = 0.2 - 2 nM) and the enantiomers of buspirone hybrid **8** displayed *in vivo* anxiolytic activity in pigeon punished responding (0.06 mg / kg i.m), and drug discrimination (0.3 mg / kg i.m. versus 8-OH-DPAT). The dextrorotatory enantiomer of **8** is under clinical investigation, S 204099 (53).



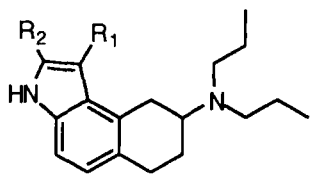
8 n = 3 R = 

9 n = 3 R = 

10 n = 1 R = 

11 n = 3 R = 

A series of C-1 substituted analogues (**12**-**14**) of the previously disclosed cyano tetrahydro-benz[e]indole derivative (**15**) was reported, with **12** having K_i = 1.8 nM for the 5-HT$_{1A}$ receptor, and 54% oral bioavailability in the rat (54,55).



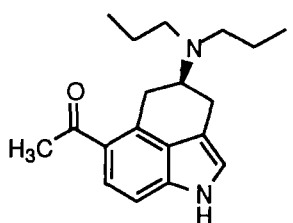
12 R_1 = CN R_2 = H

13 R_1 = Cl R_2 = H

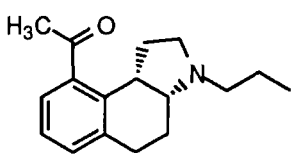
14 R_1 = CH_2CF_3 R_2 = H

15 R_1 = H R_2 = CN

Preclinical pharmacology of the potent (K_i = 0.07 nM) and selective ergoline derivative LY293284 (**16**) has been reported (56). In the pigeon conflict model, **16** was shown to be 100 times more potent than 8-OH-DPAT in increasing punished responding. Acetyl derivative **17** is also reported to be a potent (K_i = 0.4 nM) 5-HT$_{1A}$ full agonist (57).



16



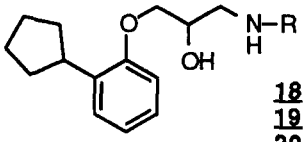
17

A computational study utilizing the Compass algorithm to generate a binding site model of the 5-HT$_{1A}$ receptor, based on 20 5-HT$_{1A}$ agonists of two chemotypes, has been recently reported. The model constructed predicted the affinities and bioactive conformation of 35 compounds, many of which contained novel structural features (58).

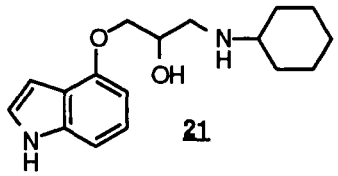
SELECTIVE 5-HT$_{1A}$ ANTAGONISTS

A comprehensive review of 5-HT$_{1A}$ receptor partial agonists and antagonists has been published (59). Since this review, novel derivatives in the aryloxypropanolamine, aminoethoxyfluorochroman and arylpiperazine families of compounds have been reported.

Aryloxypropanolamine Derivatives - Several 5-HT_{1A} antagonists and partial agonists based upon various beta-adrenoceptor antagonists have been reported. The penbutolol analogues (**18** - **20**) possess high affinity for the 5-HT_{1A} receptor while displaying up to 200-fold selectivity over the beta-adrenoceptors.

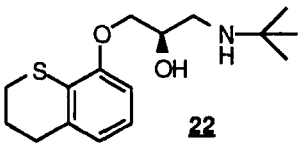


18 R = cyclopentyl
19 R = cycloheptyl
20 R = cyclooctyl



21

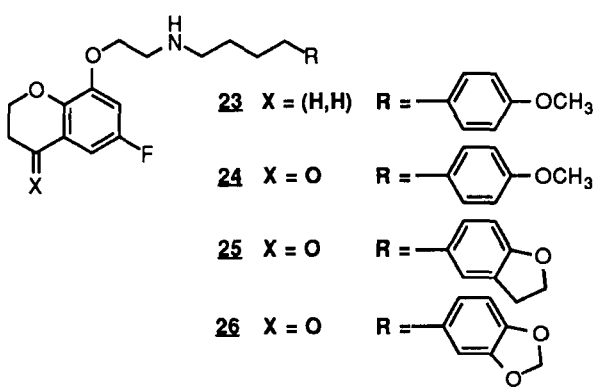
In general, increasing the ring size or steric bulk of the amino grouping increases 5-HT_{1A} selectivity relative to the beta-adrenoceptors. The cyclohexyl pindolol derivative **21** also displayed selectivity for the 5-HT_{1A} receptor (IC_{50} = 2.6 nM for 5-HT_{1A} and 26 nM for beta, vs pindolol whose IC_{50} values are 12 and 8 nM respectively) (60,61). Thiochroman beta-adrenoceptor antagonist (-)- tertatolol (**22**) shows 20-fold selectivity for the 5-HT_{1A} over the 5-HT_{1B} receptor in rat hippocampus, with no significant affinity for 5-HT_{1D}, 5-HT_3 or alpha-adrenergic receptors, or the 5-HT uptake site. Pretreatment with **22** significantly reduced the inhibitory effect of 8-OH-DPAT on the firing rate of dorsal raphe nucleus 5-HT neurons (62).



22

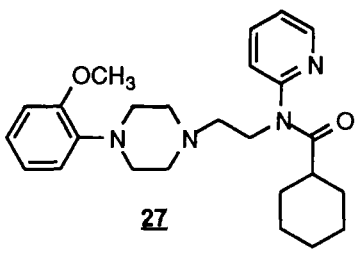
A molecular modeling study of beta-adrenoceptor derived 5-HT_{1A} partial agonists, utilizing comparative molecular field analysis (CoMFA), demonstrated the importance of steric parameters for 5-HT_{1A} affinity, and postulates that occupancy of a secondary hydrophobic pocket in the receptor binding site is important for high affinity binding (63). A three-dimensional quantitative structure-activity relationship (3-D QSAR) study of a series of 5-HT_{1A} full and partial agonists has been utilized to correlate structure with intrinsic activity (64).

Aminoethoxyfluorochromans - A series of 6-fluorochroman derivatives (**23** - **26**) has been reported which exhibits nanomolar affinity (K_i = 2- 4 nM) for the 5-HT_{1A} receptor with moderate selectivity relative to the alpha$_1$ and dopamine D_2 receptors. Compounds **23**- **26** antagonized 8-OH-DPAT - induced behavioral syndrome in rats (65).



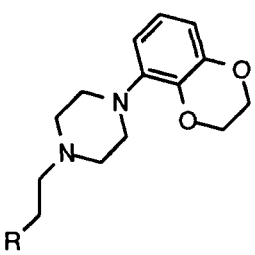
23 X = (H,H) R = OCH_3
24 X = O R = OCH_3
25 X = O R =
26 X = O R =

Arylpiperazines - (*S*)-WAY-100135 (**4**) and WAY-100635 (**27**) are the first compounds to display selective antagonism of both pre- and postsynaptic 5-HT$_{1A}$ receptors. Compound **27** displayed dose-dependent blockade of the elevation of corticosterone induced by 8-OH-DPAT with a minimum effective dose of 10 µg / kg (66,67). SAR and molecular modeling studies of derivatives related to **4** concluded that amides with large N-substituents have higher 5-HT$_{1A}$ affinity, and while the amide group is important for *in vivo* activity, hydrogen bonding by the amide is not an important binding interaction (68). Radioligands, photoaffinity labels and irreversible alkylating agents based on **27** have been reported (69,70).

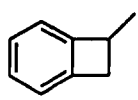


27

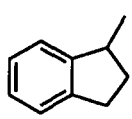
An additional series of arylpiperazines, the benzodioxopiperazine derivatives S 14489, S 15535, and S 15931 (**28**-**30**, respectively) display postsynaptic 5-HT$_{1A}$ antagonist activity while acting as agonists at the 5-HT$_{1A}$ autoreceptors with pK$_i$ values of 9.2, 8.8, and 8.9, respectively (71).

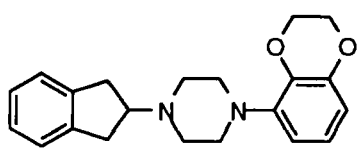


28 R =



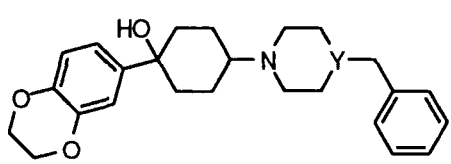
30 R =





29

Aryl substituted piperazinyl and piperidinyl cyclohexanol derivatives (**31** ,**32**) are selective 5-HT$_{1A}$ antagonists (IC$_{50}$ = 9.9 nM for **32**) which are devoid of affinity for the dopamine D$_2$ receptor, and have displayed activity in a rat social interaction task at doses of 0.001 to 0.01 mg / kg s.c. (72).



31 Y = CH

32 Y = N

Conclusions - Indirect modulation of serotonergic neurotransmission with SSRIs (e.g., fluoxetine) has been proven to have important therapeutic effects in a number of neuropsychiatric disorders. Direct interactions with the 5-HT$_{1A}$ receptor *via* selective agonists or antagonists may also have beneficial effects in a large number of diseases. Selective 5-HT$_{1A}$ full agonists have been developed and early clinical trials have shown them to be efficacious for anxiety and depression. Selective 5-HT$_{1A}$ antagonists have also been described and may be beneficial in depression, anxiety, Alzheimer's disease and prostate cancer. The progression of 5-HT$_{1A}$ agonists and

antagonists through preclinical trials and ultimately to clinical tests will lead to a greater understanding of the basic workings of the brain and potentially to an important class of therapeutics.

References

1. B.L. Jacobs and E.C. Azmitia, Physiol. Rev., 72, 165 (1992).
2. B.L. Jacobs and C.A. Fornal, Trends Neurosci., 16, 346 (1993).
3. J.M. Launay, J. Callebert, D. Bondoux, S. Loric and L. Maroteaux, Cell. Mol. Biol., 40, 327 (1994).
4. R.L. Roth, Ann. Clin. Psychiatry, 6, 67 (1994).
5. D. Hoyer, D.E. Clarke, J.R. Fozard, P.R. Hartig, G.R. Martin, E.J. Mylecharane, P.R. Saxena and P.P.A. Humphrey, Pharmacol. Rev., 46, 2 (1994).
6. M. Teitler and K. Herrick-Davis, Crit. Rev. Neurobiology, 8, 175 (1994).
7. F. Saudou and R. Hen, Neurochem. Int., 25, 503 (1994).
8. S.J. Peroutka, Synapse, 18, 241 (1994).
9. A.G. Romero and R.B. McCall, Annu. Rev. Med. Chem., 27, 21 (1992).
10. R.J. Thielen and A. Frazer, Life Sci., 56, PL 163 (1995).
11. R.W. Fuller, Neuropsychopharmacology, 3, 495 (1990).
12. J.S. Sprouse and G.K. Aghajanian, Synapse, 1, 3 (1987).
13. M.D. Tricklebank, Trends Pharmacol. Sci., 6, 403 (1985).
14. S.D. Iversen, Neuropharmacology, 23, 15530 (1984).
15. J.E. Barrett and K.E. Vanover, Psychopharmacology, 112, 1 (1993).
16. D.S. Charney, J.H. Krystal, P.L. Delgado and G.H. Heninger, Annu. Rev. Med., 41, 437 (1990).
17. R.D. Chiaie, P. Pancheri, M. Casacchia, P. Stratta, G.D. Kotzalidis and M. Zibellini, J. Clin. Psychopharmacol., 15, 12 (1995).
18. R.J. Rodgers, J.C. Cole and A. Davies, Pharmacol. Biochem. Behav., 48, 959 (1994).
19. P.F. Curle, E. Mocaer, P. Renard and B. Guardiola, Drug Dev. Res., 32, 183 (1994).
20. S.E. File and N. Andrews, Behav. Pharmacol., 5, 99 (1994).
21. L.D. Bradford, Abstracts, Am. Coll. Neuropsycopharmacol., San Juan, Peurto Rico, 167 (1994).
22. H.G.M. Westenberg and J.A. Den Boer, Pharmacopsychiatry, 26, 30 (1993).
23. D.J. Bill, A. Fletcher, Br. J. Pharmacol., 111, 151P (1994).
24. R.J. Rodgers and J.C. Cole, Eur. J. Pharmacol., 261, 321 (1994).
25. J.-L. Moreau, G. Griebel, F. Jenck, J.R. Martin, U. Widmer and W.E. Haefely, Brain Res. Bull., 29, 901 (1992).
26. P.M. Whitaker-Azmitia and E.C. Azmitia, Perspect. Dev. Neurobiol., 2, 233 (1994).
27. P.M. Whitaker-Azmitia, C. Clarke and E.C. Azmitia, Synapse, 4, 201 (1993).
28. P.T. Francis, N.R. Sims, A.W. Procter and D.M. Bowen, J. Neurochem., 60, 1589 (1993).
29. P.T. Francis, M.N. Pangalos, R.C.A. Pearson, D.N. Middlemiss, G.C. Stratmann and D.M. Bowen, J. Pharmacol. Exp. Ther., 261, 1273 (1992).
30. D. A. McCormick and A. Williamson, Proc. Natl. Acad. Sci. USA, 86, 8090 (1989).
31. D.M. Bowen, P.T. Francis, A.W. Procter, J.V. Halliwell, D.M.A. Mann and D. Neary, Ann. Neurol., 32, 112 (1992).
32. J.B. Lucot and G.H. Crampton, Pharmacol. Biochem. Behav., 33, 627 (1989).
33. J.B. Lucot, Eur. J. Pharmacol., 253, 53 (1994).
34. P.A. Abrahamsson, L.B. Wadstrom, J. Alumets, S. Falkner and L. Grimehius, Pathol. Res. Pract., 181, 675 (1986).
35. N.M. Hoosein, M.G. Bandyk, G.L. Nicolson, C.J. Logothesis and L.W.K. Chung, J. Urol., 149, 479A (1993).
36. M. Abdul, P.E. Anezinis, C.J. Logothetis and N.M. Hoosein, Anticancer Res. 14, 1215 (1994).
37. R.W. Fuller, Life Sci., 55, 163 (1994).
38. P. Blier, C. de Montingny and Y. Chaput, J. Clin. Psychopharmacol., 7, 24S (1987).
39. C. de Montigny, Y. Chaput and P. Blier, J. Clin. Psychiatry, 51, 4 (1990).
40. P. Blier and C. de Montigny, Trends Pharmacol. Sci., 15, 220 (1994).
41. S. Hjorth and S.B. Auerbach, Neuropharmacol., 33, 331 (1994).
42. T. Jolas, S. Haj-Dahmane, E.J. Kidd, X. Langlois, L. Lanfumey, C.M. Fattaccini, V. Vantalon, A.M. LaPorte, J. Adrien, H. Gozlan and M. Hamon, J. Pharmacol. Exp. Ther., 268,1432 (1994).
43. R. Invernizzi, M. Bramante and R. Samanin, Eur. J. Pharmacol., 260, 243 (1994).
44. J. J. Rutter, C. Gundlah and S.B. Auerbach, Neurosci. Lett., 171, 183 (1994).
45. R.W. Fuller, K.W. Perry, S.K. Hemrick-Luecke and E. Engleman, J. Pharm. Pharmacol., 33, in press (1995).
46. S. Hjorth, J. Neurochem., 60, 776 (1994).
47. F. Artigas, V. Perez and E. Alvarez, Arch. Gen. Psychiatry, 51, 248 (1994).
48. P. Blier and R. Bergeron, J. Clin. Psychopharmacol., 15, in press (1995).
49. M. Ansseau, W. Pitchot, A.G. Moreno, J. Wauthy and P. Papart, Human Psychopharmacol, 8, 279 (1993).

50. P. Grof, R. Joffe, S. Kennedy, E. Persad, J. Syrotiuk and D. Bradford, Int. Clin. Psychopharmacol., 8, 1676 (1993).
51. For two recent perspectives: R.A. Glennon, M. Dukat, Pharmacol. Biochem. Behav. 40, 1009 (1991); D.L. Nelson, Pharmacol. Biochem. Behav. 40, 1041 (1991).
52. C.-H. Lin, S.R. Haadsma-Svensson, R.A. Lahti, R.B. McCall, M.F. Piercy, P.J.K.D. Schreur, P.F. VonVoigtlander, C.G. Chidester, J. Med. Chem., 36, 671 (1993).
53. T. Podona, B. Guardiola-Lemaitre, D.-H. Caignard, G. Adam, B. Pfeiffer, P. Renard, G. Guillaumet , J. Med. Chem., 37, 1779 (1994).
54. A..G. Romero, J.A. Leiby, R.B. McCall, M.F. Piercy, M.W. Smith, F. Han, J. Med. Chem., 36, 2066 (1993).
55. P. Stjernlof, T. Elebring, J. Nilsson, B. Andersson, S. Lagerkvist, K. Svensson, A. Ekman, A. Carlsson, H. Wikstrom, J. Med. Chem., 37, 3263 (1994).
56. M.M. Foreman, R.W. Fuller, K. Rasmussen, D.L. Nelson, D.O. Calligaro, L. Zhang, J.E. Barrett, R.N. Booher, C.J. Paget, Jr., M.E. Flaugh, J. Pharmacol. Exp. Ther., 270, 1270 (1994).
57. S.R. Haadsma-Svensson, K. Svensson, N. Duncan, M.W. Smith, C.-H. Lin, J. Med. Chem., 38, 725 (1995).
58. A.N. Jain, N. L. Harris, J.Y. Park, J. Med. Chem., 38, 1295 (1995).
59. I.A. Cliffe, A. Fletcher, Drugs Fut., 18, 631 (1993).
60. E.E. Beedle, D.W. Robertson, D.T. Wong, US Patent 5,013,761 (1991).
61. D.T. Wong, D.N. Mayle, N.W. DeLapp, D.O. Calligaro, D.W. Robertson, Abstracts, Soc. Neurosci. 20,1542 (1994).
62. S. Prisco, A. Cagnotto, D. Talone, A. Deblasi, T. Mennini, E. Esposito, J. Pharmacol. Exp. Ther., 265 , 739 (1993).
63. M . Langlois, B. Bremont, D. Rousselle, F. Gaudy, Eur. J. Pharmacol. Mol. Pharmacol. Sect., 188, 77 (1993)
64. A. Agarwal, E.W. Taylor, J. Computational Chem., 14, 237 (1993).
65. T. Yasunaga, T. Kimura, R. Naito, S. Tsukamoto, F. Wanibuchi, T. Yamaguchi, T. Mase, Abstracts, A..C.S. 209 th Natl. Meeting, Anaheim, CA, April 2-6, MEDI No. 078 (1995).
66. A. Fletcher, D.J. Bill, S.J. Bill, I.A. Cliffe, G.M. Dover, E.A. Forster, J.T. Haskins, D. Jones, H.L. Mansell, Y. Reilly, Eur. J. Pharmacol, 237, 283 (1993).
67. A. Fletcher, D.J. Bill, I.A. Cliffe, E.A. Forster, D. Jones, Y. Reilly, Proc. Brit. Pharmacol. Soc., 112, 91P (1994).
68. S.J. Bill, I.A. Cliffe, G.M. Dover, A. Fletcher, E.A. Forster, A.D. Ifill, H.L. Mansell, W. Pitt, Y. Reilly, A.C. White, Abstracts, A..C.S. 209th Natl. Meeting, Anaheim, CA, April 2-6, MEDI No. 059 (1995).
69. Z.-P. Zhuang, M.-P. Kung, H.F. Kung. J. Med. Chem. 37, 1406 (1994).
70. Z.-P. Zhuang, M.-P. Kung, S. Chumpradit, M. Mu, H.F. Kung, J. Med. Chem. 37, 4572. (1994).
71. M.J. Millan, H. Canton, A. Gobert, F. LeJeune, J.-M. Rivet, K. Bervoets, M. Brocco, P. Widdowson, T. Mennini, V. Audinot, L. Verriele, H. Gressier, J.-L. Peglion, J. Pharmacol. Exp. Ther., 268, 337 (1994).
72. R.J. Mattson, J.D. Catt. Eur. Pat. Appl. EP 0546583 A (1993).

Chapter 2. Centrally Acting Analgesics

Allen B. Reitz, Michele C. Jetter, Kenneth D. Wild, and Robert B. Raffa
Drug Discovery Research
The R.W. Johnson Pharmaceutical Research Institute
Spring House, PA 19477-0776

Introduction - There continues to be a great need for new agents to lessen the sensation of pain, especially chronic pain, which is presently undertreated. Several important developments related to the discovery of new analgesics have emerged since the last review in *Annual Reports in Medicinal Chemistry* (1). For example, each of the major opioid receptor types has been cloned and expressed in cell culture assay systems adaptable to high-throughput screening. Subtypes of each of these receptors have been characterized and each is a distinct molecular target for drug discovery. There has also been a number of significant advances involving a variety of non-opioid analgesic mechanisms. Further, the central activity of 'peripherally-acting' non-steroidal antiinflammatory drugs (NSAIDs) and the peripheral action of 'centrally-acting' opioids have become better appreciated. In the clinical area, alternative routes of administration such as oral morphine, nasal spray, transdermal patch, spinal administration and patient-controlled analgesia (PCA) have become increasingly utilized, particularly for chronic pain (2). Two noteworthy drugs are an injectable NSAID (Toradol® ketoralac), which has achieved clinical favor, and Ultram® tramadol HCl (1), the first centrally-acting oral analgesic approved in the U.S. in ten years. The present chapter covers highlights of the general scientific and patent literature on centrally-acting analgesics during 1994, with lesser coverage of important findings during 1990-1993. The reader is also referred to recent comprehensive reviews (3-8) and to the "Current Research in Analgesia" update series found in *Analgesia*.

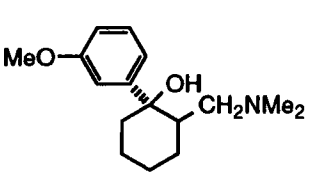
OPIOID ANALGESICS

Opioids prevail as the major class of clinically-available centrally-acting analgesics. All three opioid receptors (μ, δ and κ) have been shown to be coupled *via* inhibitory G-protein (pertussis-toxin sensitive) transduction mechanisms, and have been cloned and expressed (9-16). The first to be cloned was the mouse δ opioid receptor (mDOR) which exhibits δ-receptor binding selectivity and naloxone-reversible inhibition of forskolin-stimulated adenylyl cyclase activity when expressed in transfected COS cells (10). The μ and κ opioid receptors display sequence homology with mDOR and hDOR. Generally good correlation has been found between visualization of receptor mRNA and receptor distribution by autoradiography, suggesting local receptor synthesis at these sites and receptor transport in cases where mismatches occur (17-22). The demonstration of opioid receptor mRNA co-localization with neurotransmitters helps explain certain non-analgesic opioid characteristics such as effects on locomotor activity. The relative order of mRNA expression in the ascending and descending pain pathways has been found to be μ > κ > δ (22). Each of the three opioid receptors have been divided further into subtypes.

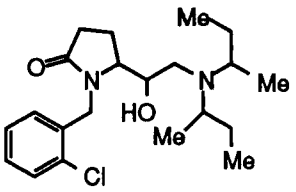
μ-Agonists - Novel pyrrolidinone **2** has been reported as a μ-selective agonist (23). All 12 possible stereoisomers were synthesized: the *S,S,R,R* isomer has high affinity and good selectivity for μ-receptors (ratios of Ki values: κ/μ 250 and δ/μ 1060), and *in vivo* antinociceptive activity. Remifentanil (3) is a newer member of the fentanil series of opioid analgesics (24). It is a selective μ-opioid ligand with a rapid onset and short duration of action and is targetted to providing analgesia during anesthesia. Functionally distinct subtypes of μ-opioid receptors have been proposed (25,26). One

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

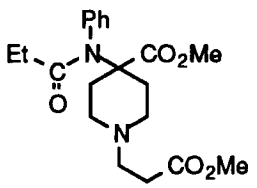
subtype (μ_1) has been associated with antinociception, whereas respiratory depression (27), constipation (28) and physical dependence (29) have been attributed to the other (μ_2). Meptazinol (4), a synthetic hexahydroazepine derivative, has modest selectivity for the μ_1 receptor subtype, but also displays significant anticholinergic activity (30).



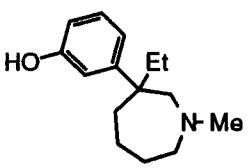
1



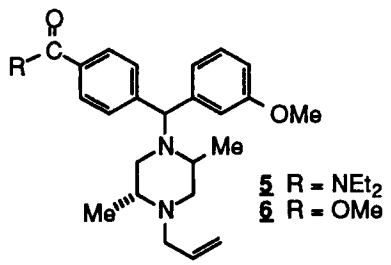
2



3



4

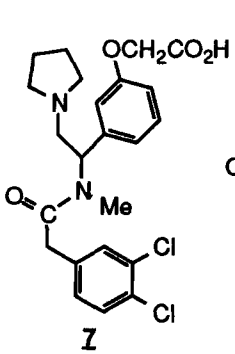


δ-Agonists - A human δ opioid receptor (hDOR) has been cloned and identified by sequence homology to other δ receptors and by its ligand recognition profile (31,32). BW373U86 (5) is a relatively selective δ agonist which produces antinociception in a variety of animal models, but was found to produce convulsions in mice and primates (33-35). SNC80, the (+) enantiomer of 6, has been reported to be essentially devoid of convulsive activity (36,37). Compound 6 displays good δ binding affinity and >2000 fold selectivity relative to the μ receptor, making it the most selective non-peptide δ agonist reported (36). There are at least two δ receptor subtypes (38-44). Oligodeoxynucleotide antisense studies suggest that mDOR corresponds to that pharmacologically classified as δ_2 (43).

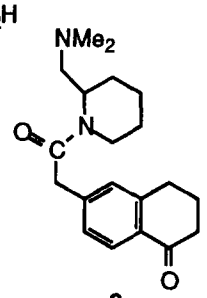
κ-Agonists - Although some currently-used drugs possess affinity for κ-receptors (e.g., pentazocine, nalbuphene and butorphanol), their pharmacology includes binding to other opioid receptor types (e.g. μ) or mixed agonist/antagonist properties. κ Agonists described to date appear to have less overall clinical efficacy than μ agonists, and possess a tendency to produce dysphoric or psychotomimetic side effects at high doses. In preclinical models, κ-agonist-induced antinociception is associated with less respiratory depression, dependence liability and withdrawal signs than traditional μ agonists, although κ-agonists have been evaluated clinically and then later withdrawn. However, the postulated existence of multiple κ-receptor binding sites (45) raises the hope that agents selective for one of these subtypes would be superior (45).

A κ agonist has been reported (7) that does not cross the blood-brain barrier, but does produce analgesia (46,47). Compound 8 displays *in vivo* antinociceptive activity and moderate affinity for κ receptors, along with reduced liability for κ-related side effects such as motor impairment, sedation, and diuresis (48). Furanylpiperazine 9 is the optimal member of a related series with *in vivo* antinociception and selective κ receptor binding (49). Compound 10 displays high affinity κ binding with good *in vivo* activity (50).

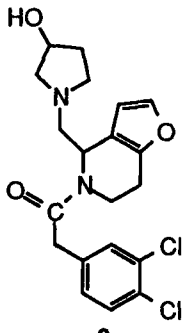
Several sets of affinity ligands have been prepared specifically for κ receptors (51-53). Of these, compound **11** and the *S*-enantiomer of compound **12** produced long-lasting analgesia in mice, suggesting irreversible receptor inactivation (52,53).



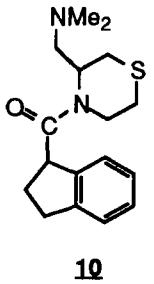
7



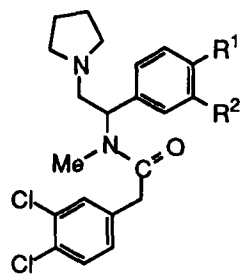
8



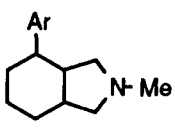
9



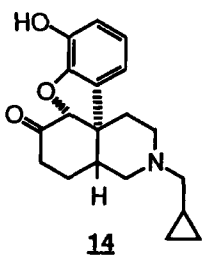
10



11 R^1 = H, R^2 = NCS
12 R^1 = NCS, R^2 = H



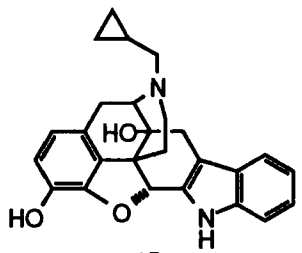
13



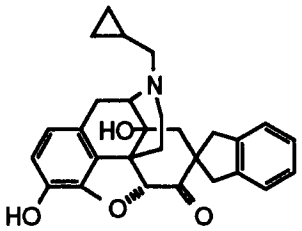
14

Mixed Opioid Ligands - Tramadol (**2**) binds to opioid receptors and inhibits the neuronal reuptake of norepinephrine and serotonin (54-57). Tramadol is marketed as a racemic mixture. The opioid binding and neurotransmitter reuptake inhibition are found predominantly in the separate enantiomers which interact synergistically in several *in vivo* antinociceptive models (58). Aryl-substituted octahydroisoindoles **13** are structurally related to deshydroxytramadol and were found to have *in vivo* analgesic activity (59). *trans*-Ketone **14** displays significant opioid receptor affinity and *in vivo* antinociception, and is relatively devoid of sigma binding (60). The indolic portion of naltrindole (**15**) is purported to play a key role in interaction with the address subsite of the δ receptor and is an application of the address-message concept. Further extension of this approach led to spiro structure **16** which displayed potent δ *in vitro* affinity and *in vivo* antinociceptive activity (61). Indolic derivatives of 5-(3-hydroxyphenyl)morphans such as **17** displayed a >180 fold increased affinity for the δ receptor (62). Aralkyl ethers of naltrexol were prepared to examine the effect of additional steric bulk at the 6-position. The 6α-O-benzyl and the 6β-O-benzyl compounds had the most potent opioid receptor affinities (63).

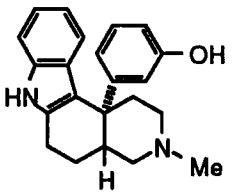
N-Oxides of opioids such as tramadol (**2**) have been found to be useful prodrug derivatives (2,64-66). Delayed systemic availability of the parent analgesic drugs may allow for less frequent dosing to achieve the same therapeutic effect.



15



16

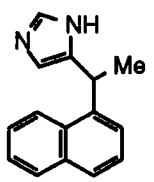


17

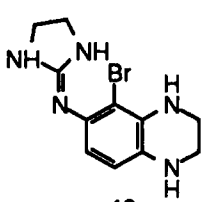
The first cyclic peptide analog with mixed μ agonist/δ antagonist properties was reported (67). Other constrained and modified peptide derivatives were prepared to continue probing conformational requirements for opioid receptor binding (68-73). Polyethyleneglycol (PEG) hybrids of enkephalin analogs were found to produce prolonged antinociception comparable to morphine (74), and showed increased stability to metabolic inactivation.

NON-OPIOID ANALGESICS

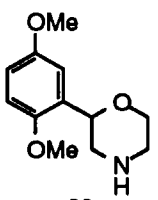
α_2-Adrenoceptor Agonists – Compounds with α_2-adrenoceptor agonist activity are analgesic in animals and humans, and are expected to have less abuse liability than opioids (75-79). Three receptor subtypes have been cloned and are termed α_{2A}, α_{2B}, and α_{2C}, based on their pharmacology (80,81). The subtypes are also named α_2-C10, α_2-C2, and α_2-C4, respectively, according to their human chromosomal localization (82). Correlation of the binding of a large number of α_2 agonists with antinociceptive activity in the rat suggests the α_{2A} receptor mediates analgesia in that species, but does not formally rule out an involvement of the other subtypes (83). Compounds which do not bind to non-adrenergic imidazoline receptors are expected to have fewer cardiovascular side-effects (84). Structure-activity relationships for α_2-adrenoceptor binding have been reviewed (8,85). 4-Substituted imidazoles and 2-substituted imidazolines continue to be prominent substructures found in many of the reported ligands (86-88). Compounds **18** and **19** bear fused bicyclic rings similar to that found in brimonidine (UK 14,304), which is presently being evaluated clinically for reduction of intraocular pressure, another α_2-mediated effect (86,87,89). 2-Amino-2-oxazoline derivatives form the basis for an additional series of α_2-adrenoceptor ligands (88). Aryl-substituted morpholines such as **20** are α_2-agonists (90). Isothiocyanate **21** was prepared as an affinity probe for α_2-receptor binding (91).



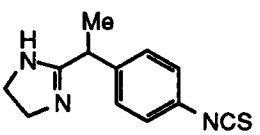
18



19



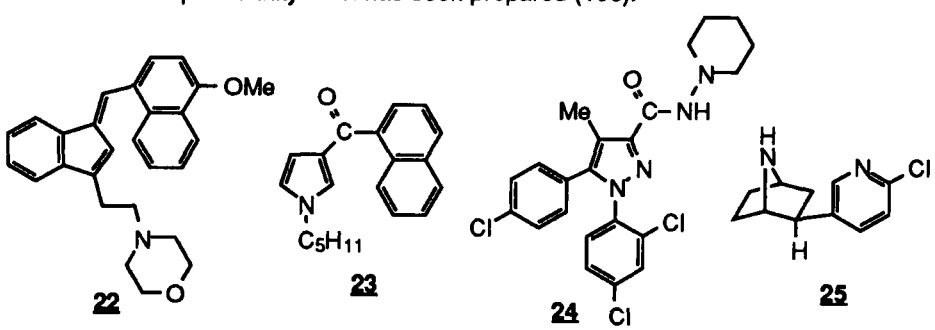
20



21

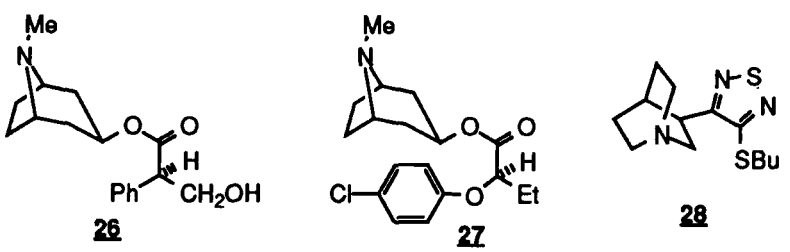
Cannabinoids – Cannabinoid receptors have been identified in the CNS, and they are potential targets for non-opioid analgesia (92,93). Human cannabinoid receptor (hCBR) cDNA has 98% amino acid sequence identity to the rat cDNA (94). The brain cannabinoid receptor (CB_1) belongs to the G-protein family and is believed to inhibit adenylyl cyclase activity through activation of a G_i protein (95). A second cannabinoid receptor (CB_2) has been cloned and has been termed the 'peripheral cannabinoid receptor' because of its localization primarily in the imune system (96). The low overall

sequence homology (44%) between CB_1 and CB_2 suggests subtype-selective compounds could be discovered. There is also the possible existence of other receptor subtypes not detected using conventional screening methods (97). The N-(2-hydroxyethyl)amide of arachidonic acid is the putative endogenous ligand for cannabinoid receptors, and it produces significant antinociception in tests predictive of clinical efficacy (98,99). Cannabinomimetic compounds produce non-opioid (naloxone-insensitive) antinociception *via* spinal or supraspinal sites in rodents, probably through the CB_1 receptor (100-102). Cannabinoid receptor affinity paralleled antinociceptive activity for a series of indene derivatives (e.g. **22**) of pravadoline (103). 3-Acylpyrrole **23** displayed cannabinoid activity comparable to tetrahydrocannabinol (104). A cannabinoid receptor antagonist (**24**) has been described (105). The first irreversible cannabinoid receptor affinity label has been prepared (106).



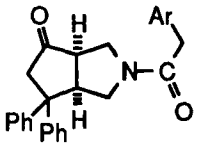
Cholinergic Agents - Epibatidine (**25**) is a nicotinic cholinergic agonist which produces potent antinociception that is not blocked by the opioid antagonist naloxone or by hexamethonium, but is attenuated by pretreatment with the nicotinic cholinergic receptor antagonist mecamylamine (107,108). Three new chemical syntheses of epibatidine were reported in 1994, and both enantiomers have been shown to have antinociceptive activity (109-112).

(*R*)-(+)-Hyoscyamine (**26**) has antinociceptive activity resulting from an increased acetylcholine release secondary to antagonism of central muscarinic autoreceptors (113,114). Related 2-phenoxypropionic acids such as **27** were found to have potent antinociceptive and cognition-enhancing properties, possibly by antagonizing presynaptic M_2 receptors (114). Additionally, muscarinic agonists such as **28** produced antinociception (115).

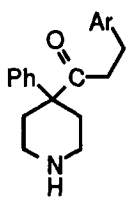


Galanin Ligands - Galanin is a 29 to 30 (human) amino acid neuropeptide found in a variety of peripheral and central tissues (116). Galanin depresses nociceptive reflex in newborn rat spinal cord and is antinociceptive to mechanical stimuli when injected spinally to rats (117,118). Galanin also potentiates the action of morphine and blocks the nociceptive effect of substance P and calcitonin gene related protein pre- and post-synaptically (119). The availability of the human cloned receptor now makes galanin a target for high-throughput screening (120).

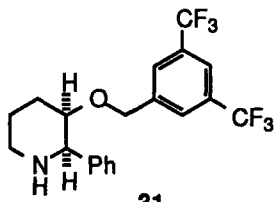
Tachykinin Antagonists - *The potential of neurokinin receptor antagonists as anti-nociceptive agents has been reviewed (5).* A variety of neurokinin receptor ligands have been reported (121-137), including pyrrolidine **29** and 4-phenylpiperidine **30**. Compound **31** is a member of a large class of related structures in which the quinuclidine moiety found in the original non-peptide Substance P antagonists has been simplified to a piperidine ring bearing pendant aryl substitution (123).



29



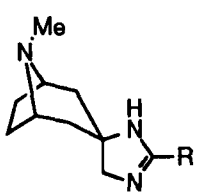
30



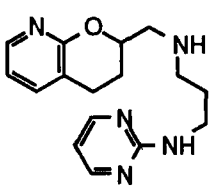
31

Bradykinin Antagonists - Inhibiting the formation of bradykinin or blocking its action is expected to produce non-opioid analgesia (7,138,139). Since bradykinin and the related decapeptide kalliden are generated from precursors (kininogens) by the proteolytic action of serine proteases (kallikreins), kallikrein inhibitors might also be useful as analgesics. Bradykinin-induced nociceptive effects are mediated through cell-surface BK_1 and BK_2 G-protein-coupled receptors (140,141). The peptidic bradykinin antagonist NPC-567 was tested clinically with disappointing results (139). *The first reported series of non-peptide BK_2 receptor antagonists was based on* bisphosphonium salts found initially by broad screening, but analgesic evaluation was not disclosed (142,143).

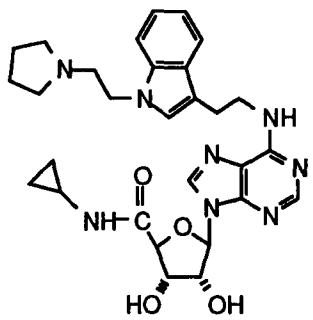
Cholecystokinin Antagonists - The CCK antagonist MK-329 was progressed to clinical trials for chronic pain in patients with advanced pancreatic cancer, but clinical efficacy was not demonstrated (144).



32



33



34

Serotonergic Ligands - There are many serotonin (5-HT) receptor subtypes (5,145). Sumatriptan is a putative selective $5\text{-}HT_{1D}$ agonist which is used for the treatment of migraine headaches (146). Several series of $5\text{-}HT_3$ antagonists have been reported, including **32**, which may be useful in antimigraine therapy (147,148). Dihydropyranopyridines such as **33** have $5HT_1$-like agonist activity and are claimed to prevent cephalic pain, especially migraine (149).

Adenosine Agonists - Enhancement of adenosine release by morphine suggests a potential role for adenosine in analgesia (150). A series of adenosine derivatives such as **34** displayed good *in vivo* antinociceptive activity, which correlated well with A_1 receptor affinity (150).

Conclusions - The relief of chronic pain is the goal of medicinal chemistry research in the area of analgesia. The present availability of human cloned opioid receptor subtypes allows for the directed synthesis of subtype-selective agents which could have morphine-like antinociception, but without the abuse liability, sedation, and respiratory depression that restrict the use of current opioids. Non-opioid analgesics have the inherent advantage of lacking opioid-related side-effects. As with opioids, receptor sub-type selective agents, such as for α_2-adrenoceptor agonists, may provide the best combination of analgesia relative to limiting side-effects. Testing of compound libraries in high-throughput screening involving the appropriate cloned receptors or *in vitro* assays has enormous potential, and could facilitate the discovery of new analgesic drugs.

References

1. S.M. Evans, G.R. Lenz and R.A. Lessor, Annu. Repts. Med. Chem., 25, 11 (1990).
2. H.L. Fields and J.C. Liebeskind, Eds., "Progress in Pain Research and Management. Vol. 1 - Pharmacological Approaches to the Treatment of Chronic Pain: New Concepts and Critical Issues", IASP Press, Seattle, 1994.
3. G.F. Gebhart, D.L. Hammond and T.S. Jensen, Eds., "Progress in Pain Research and Management. Vol. 2 - Proceedings of the 7th World Congress on Pain", IASP Press, Seattle, 1994.
4. G.F. Gebhart, J.D. Haddock, A. Jacox, N.A. Janjan, T.E. Rudy and B.S. Shapiro, Eds., "Yearbook of Pain 1994", Mosby, St. Louis, 1994.
5. S.H. Buck, Ed., "The Tachykinin Receptors", Humana Press, Totowa, N.J., 1994.
6. J.M. Besson, "Serotonin and Pain", Excerpta Medica, Amsterdam, 1990.
7. R.M. Burch, D.J. Kyle and T.M. Storman, "Molecular Biology and Pharmacology of Bradykinin Receptors", R.G. Landes Co., Austin, Texas, 1993.
8. K.D. Wild, J.B. Press and R.B. Raffa, Analgesia, 1, 15 (1994).
9. R.B. Raffa and R.P. Martinez, Eur. J. Pharmacol. 215, 357 (1992).
10. C.J. Evans, D.E. Keith Jr., H. Morrison, K. Magendzo and R.H. Edwards, Science, 258, 1952 (1992).
11. B.L. Kieffer, K. Befort, C. Gaveriaux-Ruff and C.G. Hirth, Proc. Natl. Acad. Sci., 89, 12048 (1992).
12. F. Meng, G.X. Xie, R.C. Thompson, A. Mansour, A. Goldstein, S.J. Watson and H. Akil, Proc. Natl. Acad. Sci., 90, 9954 (1993).
13. K. Yasuda, K. Raynor, H. Kong, C.D. Breder, J. Takeda, T. Reisine and G.I. Bell, Proc. Natl. Acad. Sci., 90, 6736 (1993).
14. Y. Chen, A. Mestek, J. Liu, J.A. Hurley and L. Yu, Mol. Pharmacol., 44, 8 (1993).
15. J.B. Wang, Y. Imai, C.M. Eppler, P. Gregor, C.E. Spivak and G.R. Uhl, Proc. Natl. Acad. Sci., 90, 10230 (1993).
16. R.C. Thompson, A. Mansour, H. Akil and S.J. Watson, Neuron, 11, 903 (1993).
17. A. Mansour, C.A. Fox, R.C. Thompson, H. Akil and S.J. Watson, Brain Res., 643, 245 (1994).
18. J.M. Delfs, K. Haeyoung, A. Mestek, Y. Chen, L. Yu, T. Reisine and M.F. Chesselet, J. Comp. Neurol., 345, 46 (1994).
19. M.K.H. Schäfer, M. Bette, H. Romero, W. Schwaeble and E. Weihe, Neurosci. Lett., 167, 137 (1994).
20. K. Maekawa, M. Minami, K. Yabuuchi, T. Toya, Y. Katao, Y. Hosoi, T. Onogi and M. Satoh, Neurosci. Lett., 168, 97 (1994).
21. R.J. Dado, P.Y. Law, H.H. Loh and R. Elde, NeuroReport, 5, 341 (1993).
22. A. Mansour, C.A. Fox, H. Akil and S.J. Watson, Trends Neurosci., 18, 22 (1995).
23. M. Napoletano, D. Della Bella, F. C. Grancini, C. Masotto, S. Ricciardi and C. Zambon, BioMed. Chem. Lett., 5, 589 (1995).
24. C. Robinson and J. Castaner, Drugs of the Future, 19, 1088 (1994).
25. C.G. Pick, R.J. Nejat and G.W. Pasternak, J. Pharmacol. Exp. Ther., 265, 166 (1993).
26. R.B. Raffa and J.J. Schupsky, Life Sci./Pharmacol. Lett., 54, PL 57 (1994).
27. G.S.F. Ling, K. Spiegel, S.H. Lockhart and G.W. Pasternak, J. Pharmacol. Exp. Ther., 232, 149 (1985).
28. J.S. Heyman, C.L. Williams, T.F. Burks, H.I. Mosberg and F. Porreca, J. Pharmacol. Exp. Ther., 245, 238 (1988).
29. G.S.F. Ling, J.M. MacLeod, S. Lee, S.H. Lockhart and G.W. Pasternak, Science, 226, 462 (1984).
30. P.J. Hoskin and G.W. Hanks, Drugs, 41, 326 (1991).
31. R.J. Knapp, E. Malatynska, L. Fang, X. Li, E. Babin, M. Nguyen, G. Santoro, E. Varga, V.J. Hruby, W.R. Roeske and H.I. Yamamura, Life Sci/Pharmacol. Lett, 54, PL463 (1994).

32. E. Malatynska, Y. Wang, R.J. Knapp, G. Santoro, X. Li, S. Waite, W.R. Roeske and H.I. Yamamura, NeuroReport, 6, 613 (1995).
33. K.D. Wild, J. McCormick, E.J. Bilsky, T. Vanderah, R.W. McNutt, K.J. Chang and F. Porreca, J. Pharmacol. Exp. Ther., 267, 858 (1993).
34. K.J. Chang, G.C. Rigdon, J.L. Howard and R.W. McNutt, J. Pharmacol. Exp. Ther., 267, 852 (1993).
35. S.D. Comer, E.M. Hoenicke, A.I. Sable, R.W. McNutt, K.J. Chang, B.R. De Costa, H.I. Mosberg and J.H. Woods, J. Pharmacol. Exp. Ther., 267, 888 (1993).
36. S.N. Calderon, R.B. Rothman, F. Porreca, J.L. Flippen-Anderson, R.W. McNutt, H. Xu, L.E. Smith, E.J. Bilsky, P. Davis and K.C. Rice, J. Med. Chem., 37, 2125 (1994).
37. E.J. Bilsky, S.N. Calderon, T. Wang, R.N. Bernstein, P. Davis, V.J. Hruby, R.W. McNutt, R.B. Rothman, K.C. Rice and F. Porreca, J. Pharmacol. Exp. Ther., 273, 359 (1995).
38. M. Sofuoglu, P.S. Portoghese and A.E. Takemori, J. Pharmacol. Exp. Ther., 257, 676 (1991).
39. M. Sofuoglu, P.S. Portoghese and A.E. Takemori, Life Sci./Pharmacol. Lett., 49, PL153 (1991).
40. Q. Jiang, A.E. Takemori, M. Sultana, P.S. Portoghese, W.D. Bowen, H.I. Mosberg and F. Porreca, J. Pharmacol. Exp. Ther., 257, 1069 (1991).
41. A. Mattia, T. Vanderah, H.I. Mosberg and F. Porreca, J. Pharmacol. Exp. Ther., 258, 583 (1991).
42. J.R. Traynor and J. Elliott, Trends Pharmacol. Sci., 14, 84 (1993).
43. J. Lai, E.J. Bilsky, R.B. Rothman, and F. Porreca, NeuroReport 5, 1049 (1994).
44. K.D. Wild, F. Porreca, H.I. Yamamura and R.B. Raffa, Proc. Natl. Acad. Sci., 91, 12018 (1994).
45. R.B. Rothman, Analgesia, 1, 27 (1994).
46. H. Kelta, V. Kayser and G. Guilbaud, Eur. J. Pharmacol., 277, 275 (1995).
47. J.S. Shaw, J.A. Carol, P. Akuck and B.G. Main, Br. J. Pharmacol., 96, 986 (1989).
48. G. Giardina, G.D. Clarke, G. Dondio, G. Petrone, M. Sbacchia and V. Vecchietti, J. Med. Chem., 37, 3482 (1994).
49. A. Naylor, D.B. Judd, D.I.C. Scopes, A.G. Hayes and P.J. Birch, J. Med. Chem., 37, 2138 (1994).
50. A. Terada, Y. Iizuka, K. Wachi and K. Fujibayashi, WO 9405646 (1994).
51. A.C. Chang, A.E. Takemori and P.S. Portoghese, J. Med. Chem., 37, 1547 (1994).
52. A.C. Chang, A.E. Takemori, W.H. Ojala, W.H. Gleason and P.S. Portoghese, J. Med. Chem., 37, 4490 (1994).
53. S.A. Weerawarna, R.D. Davis and W. Nelson, J. Med. Chem., 37, 2856 (1994).
54. E. Friderichs, W. Reimann and N. Selve, N.S. Arch. Pharmacol., 346, R36 (1992).
55. R.B. Raffa, E. Friderichs, W. Reimann, R.P. Shank, E.E. Codd and J.L. Vaught, J. Pharmacol. Exp. Ther., 260, 275 (1992).
56. B. Driessen and W. Reimann, Br. J. Pharmacol., 105, 147 (1992).
57. B. Driessen, W. Reimann and H. Giertz, Br. J. Pharmacol., 108, 806 (1993).
58. R.B. Raffa, E. Friderichs, W. Reimann, R.P. Shank, E.E. Codd, J.L. Vaught, H.I. Jacoby and N. Selve, J. Pharmacol. Exp. Ther., 267, 331 (1993).
59. R.J. Carmosin, J.R. Carson, D.C. Liotta, P. Pitis and R.B. Raffa, WO 9422823 (1994).
60. C.Y. Cheng, L.W. Hsin, M.C.Tsai, W.K. Schmidt, C. Smith and S.W. Tam, J. Med. Chem., 37, 3121 (1994).
61. P.S. Portoghese, M. Sultana, S.T. Moe and A.E. Takemori, J. Med. Chem., 37, 579 (1994).
62. C.M. Bertha, J.L. Flippen-Anderson, R.B. Rothman, F. Porreca, P. Davis, H. Xu, K. Becketts, X.Y. Cha and K.C. Rice, J. Med. Chem., 38, 1523 (1995).
63. T.D. Nelson, R.D. Davis and W.L. Nelson, J. Med. Chem., 37, 4270 (1994).
64. R.B. Raffa, C.A. Maryanoff and F.J. Villani, U.S. 5,223,541 (1993).
65. G.A. Boswell and M.J. Myers, US 4,722,928 (1988).
66. G.A. Boswell and M.J. Myers, US 4,990,617 (1991).
67. R. Schmidt, D. Vogel, C. Mrestani-Klaus, W. Brandt, K. Neubert, N.N. Chung, C. Lemieux and P.W. Schiller, J. Med. Chem., 37, 1136 (1994).
68. X. Qian, K.E. Kover, M.D. Shenderovich, B.S. Lou, A. Misicka, T. Zalewska, R. Horvath, P. Davis, E.J. Bilsky, F. Porreca, H.I. Yamamura and V.J. Hruby, J. Med. Chem., 37, 1746 (1994).
69. F.D.T. Lung, J.Ph. Meyer, G. Li, B.S. Lou, D. Stropova, P. Davis, H.I. Yamamura, F. Porreca and V.J. Hruby, J. Med. Chem., 38, 585 (1995).
71. J.P. Meyer, N. Collins, F.D. Lung, P. Davis, T. Zalewska, F. Porreca, H.I. Yamamura and V.J. Hruby, J. Med. Chem., 37, 3910 (1994).
72. A. Misicka, A.W. Lipkowski, R. Horvath, P. Davis, H.I. Yamamura, F. Porreca and V.J. Hruby, J. Med. Chem., 37, 141 (1994).
73. B.S. Pitzele, R.W. Hamilton, K.D. Kidla, S. Tsymbalov, A. Stapefeld, M.A. Savage, M. Clare, D.L. Hammond and D.W. Hansen, Jr., J. Med. Chem., 37, 888 (1994).
74. M. Maeda, K. Kawasaki, M. Takahashi, K. Nakao and H. Kaneto, Chem. Pharm. Bull., 42, 1859 (1994).
75. E.R. Butelman and J.H. Woods, J. Pharmacol. Exp. Ther., 264, 762 (1993).
76. J.M. Bernard, J.L. Hommeril, N. Passuti and M. Pinaud, Anesthesiology, 75, 577 (1991).
77. M.J. Jaakola, M. Salonen, R. Lehtinen and H. Scheinin, Pain, 46, 281 (1991).
78. M. Tryba, M. Zenz and M. Strumpf, Anesthesiology, 75, A1085 (1991).

79. H.J. McQuay, "Towards the Use of Noradrenergic Agonists for the Treatment of Pain," Int. Cong. Series - Exerpta Medica, J.M. Besson and G. Guilbaud, Eds., Exerpta Medica, p1006, New York, 1992.
80. J.W. Lomasney, W. Lorenz, L.F. Allen, K. King, J.W. Regan, T.L. Yang-Feng, M.G. Caron and R.L. Lefkowitz, Proc. Natl. Acad. Sci., 87, 5094 (1990).
81. D.B. Bylund, H.S. Blaxall, L.J. Iversen, M.G. Caron, R.J. Lefkowitz and J.W. Lomasney, Mol. Pharmacol., 42, 1 (1992).
82. D.J. Pepperl and J.W. Regan, "Handbook of Receptors and Channels", S.J. Peroutka, Ed., CRC Press, Ann Arbor, MI , 1994.
83. M.J. Millan, K. Bervoets, J.M Rivet, P. Widdowson, A. Renouard, S. Le Marouille-Girardon and A. Gobert, J. Pharmacol. Exp. Ther., 270, 958 (1994).
84. E.E. Codd, J.B. Press and R.B. Raffa, Life Sci., 56, 63 (1995).
85. R.R. Ruffolo, Jr., R. DeMarinis, M. Wise and J.P. Hieble, "The Alpha-2 Adrenergic Receptors" L.E. Limbird, Ed., Humana Press, Clifton, NJ, 1988..
86. S.S. Hong, K.J. Romstedt, D.R. Feller, F.L. Hsu, T.L. Cupps, R.A. Lyon and D.D. Miller, J. Med. Chem., 37, 2328 (1994).
87. J.R. Bagley, L.L. Brockunier, L.V. Kudzma, C.R. Pandit, D.V. Pratt, A. Galdes, T.P. Jerussi, R.A. Del Vecchio and S. Harris, J. Med. Chem., 37, 346 (1994).
88. S.A. Munk, C. Gluchowski, L. Dolby, H. Wong, J. Burke, A. Kharlamb, C. Manlapaz, E. Padillo, D. Rodgers, B. Ohta, L. Wheeler and M. Garst, BioMed. Chem. Lett., 4, 459 (1994).
89. Drugs of the Future, 19, 683 (1994).
90. C. Manera, A. Martinelli, S. Nencetti, F. Romagnoli, A. Rossello, G. Giannaccini, R. Scatizzi, P. Cozzini and P. Domiano, Eur. J. Med. Chem., 29, 519 (1994).
91. M. Slavica, L. Lei, P.N. Patil, A. Ketezy, D.R. Feller and D.D. Miller, J. Med. Chem., 37, 1874 (1994).
92. C.M. Gerard, C. Mollereau, G. Vassart and M. Parmentier, Biochem. J. 279, 129 (1991).
93. A.C. Howlett, Annu. Rev. Pharmacol. Toxicol., 35, 607 (1995).
94. C. Gerard, C. Mollereau, G. Vassart and M. Parmentier, Nucl. Acids Res., 18, 7142 (1990).
95. A..C. Howlett, D.M. Evans and D.B. Houston in "Marijuana/Cannabinoids Neurobiology and Neurophysiology", L. Murphy and A. Bartke, Eds., CRC Press, p35, 1992.
96. S. Munro, K.L. Thomas and M. Abu-Shaar, Nature, 365, 61 (1993).
97. W. Devane, L. Hanus, A. Breuer, R.G. Pertwee, L.A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger and R. Mechoulam, Science 258, 1946 (1992).
98. W.A. Devane, Trends Pharmacol. Sci., 15, 40 (1994).
99. E. Fride and R. Mechoulam, Eur. J. Pharmacol. 231, 313 (1993).
100. A.H. Lichtman and B.R. Martin, J. Pharmacol. Exp. Ther., 258, 517 (1991).
101. S.P. Welch and D.L. Stevens, J. Pharmacol. Exp. Ther., 262, 10 (1992).
102. L.S. Melvin, G.M. Milne, M.R. Johnson, B. Subramaniam, G.H. Wilken and A.C. Howlett, Mol. Pharmacol., 44, 1008 (1993).
103. V. Kumar, M.D. Alexander, M.R. Bell, M.A. Eissenstat, F.M. Casiano, S.M. Chippari, D.A. Haycock, D.A. Luttinger, J.E. Kuster, M.S. Miller, J.I. Stevenson and S.J. Ward, BioMed. Chem. Lett., 5, 381 (1994).
104. J.A.H. Lainton, J.W. Huffman, B.R. Martin and D.R. Compton, Tet. Lett., 36, 1401 (1995).
105. M. Rinaldi-Carmona, F. Barth, M. Héaulme, D. Shire, B. Calandra, C. Congy, S. Martinez, J. Maruani, G. Néliat, D. Caput, P. Ferrara, P. Soubrié, J.C. Brelière and G. Le Fur, FEBS Lett., 350, 240 (1994).
106. Y. Guo, V. Abadji, K.L. Morse, D.J. Fournier, X. Li and A. Makriyannis, J. Med. Chem., 37, 3867 (1994).
107. C. Qian, T. Li, T.Y. Shen, L. Libertine-Garahan, J. Eckman, T. Biftu and S. Ip, Eur. J. Pharmacol., 250, R13 (1993).
108. B. Badio and J. Daly, Mol. Pharmacol., 45, 563 (1994).
109. C. Szantay, Z. Kardosbalogh, I. Moldvai, C. Szantay, E. Temesvar-Major and G. Blasko, Tet. Lett., 35, 3171 (1994).
110. K. Sonokuchi, H. Nakai, M. Kawamura, N. Katsube, S. Nonaka, H. Sawaragi and N. Hamanaka, Synlett., 343 (1994).
111. K. Okoabe and M. Natsume, Chem. Pharm. Bull., 42, 1432 (1994).
112. D.W. Bonhaus, K.R. Bley, C.A. Broka, D.J. Fontana, E. Leung, R. Lewis, A. Shieh and E.H.F. Wong, J. Pharmacol. Exp. Ther., 272, 1199 (1995).
113. F. Gualtieri, G. Conti, S. Dei, M.P. Giovannoni, F. Nannucci, M.N. Romanelli, S. Scapecchi, E. Teodori, L. Fanfani, C. Ghelardini, A. Giotti and A. Bartolini, J. Med. Chem., 37, 1704 (1994).
114. F. Gualtieri, C.Bottalico, A. Calandrella, S. Dei, M.P. Giovannoni, S. Mealli, M.N. Romanelli, S. Scapecchi, E. Teodori, N. Galeotti, C. Ghelardini, A. Giotti and A. Bartolini, J. Med. Chem., 37, 1712 (1994).
115. P. Sauerberg, P.H. Olesen, M.J. Sheardown, P.D. Suzdak, H.E. Shannon, F.B. Bymaster, D.O. Calliforo, C.H. Mitch, J.S. Ward and M.D.B. Swedberg, Life Sci., 55, 807 (1994).
116. T. Bartfai, G. Fisone and U. Langel, Trends Pharmacol. Sci., 13, 312 (1990).
117. M. Yanagisawa, N. Yagi, M. Otsuka, C. Yanaihara and N. Yanaihara, Neurosci. Lett., 70, 278 (1986).

118. Y. Kuraishi, M. Kawamura, T. Yamaguchi, T. Houtani, S. Kawabata, S. Futaki, N. Fujii and M. Satoh, Pain, 44, 321 (1991).
119. Z. Wiesenfeld-Hallin, M.J. Villar and T. Hökfelt, Brain Res., 486, 205 (1986).
120. E. Habert-Ortoli, B. Amiranoff, I. Loquet, M. Laburthe and J.F. Mayaux, Proc. Natl. Acad. Sci., 91, 9780 (1994).
121. J.L. Malleron, J.F. Peyronel, P. Desmazeau, C. M'Houmadi and C. Planiol, Tet. Lett., 36, 543 (1994).
122. A.M. Macleod and K.J. Merchant, WO 950404042-A1 (1995).
123. T. Ladduwahetty, L. Keown, M.A. Cascieri and S. Sadowski, BioMed. Chem. Lett., 4, 1917 (1994).
124. A.M. MacLeod, K.J. Merchant, F. Brookfield, F. Kelleher, G. Stevenson, A.P. Owens, C.J. Swain, M.A. Cascieri, S. Sadowski, E. Ber, C.D. Strader, D.E. MacIntyre, J.M. Metzger, R.G. Ball and R. Baker, J. Med. Chem., 37, 2145 (1994).
125. B.J. Williams, M. Teall, J. McKenna, T. Harrison, C.J. Swain, M.A. Cascieri, S. Sadowski, C. Strader and R. Baker, BioMed. Chem. Lett., 4, 1903 (1994).
126. C.J. Swain, M.A. Cascieri, A. Owens, W. Saari, S. Sadowski, C. Strader, M. Teall, M.B. VanNiel and B.J. Williams, BioMed. Chem. Lett., 4, 2161 (1994).
127. T. Harrison, B.J. Williams, C.J. Swain and R.G. Ball, BioMed. Chem. Lett., 4, 2545 (1994).
128. T. Harrison, B.J. Williams and C.J. Swain, BioMed. Chem. Lett., 4, 2733 (1994).
129. S.G. Grdadolnik, D.F. Mierke, G. Byk, I. Zeltser, C. Gilon and H. Kessler, J. Med. Chem., 37, 2145 (1994).
130. D. Hagiwara, H. Miyake, N. Igari, M. Karino, Y. Maeda, T. Fujii and M. Matsuo, J. Med. Chem., 37, 2090 (1994).
131. D. Achard., A. Truchon and J.F. Peyronel, BioMed. Chem. Lett., 4, 669 (1994).
132. J.A. Lowe, III, F.E. Ewing, M. Snider, K.P. Longo, J.W. Constantine, W.S. Lebel, H.A. Woody and J. Bordner, BioMed. Chem. Lett., 4, 839 (1994).
133. J.A. Lowe, III, S.E. Drozda, S. McLean, R.T. Crawford, D.K. Bryce and J. Bordner, BioMed. Chem. Lett., 4 1153 (1994).
134. M.C. Desai, S.L. Lefkowitz, D.K. Bryce and S. McLean, BioMed. Chem. Lett., 4, 1865 (1994).
135. C.G. Bradshaw, K. Ceszkowski, G. Turcatti, I.J.M. Beresford and A. Chollet, J. Med. Chem, 37, 1991 (1994).
136. A.W.J. Cooper, H.S. Adams, P. Bell, P.M. Gore, A.B. McElroy, J.M. Pritchard, P.W. Smith and P. Ward, BioMed. Chem. Lett., 4, 1951 (1994).
137. B.A. Morgan, T.D. Gordon, P.E. Hansen and J. Singh, US 5,378,803 (1995).
138. J.M. Bathon and D. Proud, Annu. Rev. Pharmacol. Toxicol., 31, 129 (1991).
139. S.G. Farmer and R.M. Burch, Annu. Rev. Pharmacol. Toxicol., 32, 511 (1992).
140. D.J. Kyle and R.M. Burch, Curr. Opin. Invest. Drugs, 2, 5 (1993).
141. J.M. Hall, Pharmacol. Ther., 56, 131 (1992).
142. J.M. Salvino, P.R. Seoane, B.D. Douty, M.A. Awad, R.E. Dolle, W.T. Houck, D.M. Faunce and D.G. Sawutz, J. Med. Chem., 36, 2583 (1993).
143. J.M. Salvino, P.R. Seoane, B.D. Douty, M.A. Awad, D. Hoyer, T.M. Ross, R.E. Dolle, W.T. Houck, D.M. Faunce and D.G. Sawutz, BioMed. Chem. Lett., 5, 357 (1995).
144. J.L. Abbruzzese, C.F. Gholson, K. Daugherty, E. Larson, R. Dubrow, R. Berlin and B. Levin, Pancreas, 7, 165 (1992).
145. D. Hoyer, D.E. Clarke, J.R. Fozard, P.R. Hartig, G.R. Martin, E.J. Mylecharane, P.R. Saxena and P.A. Humphrey, Pharmacol. Rev., 46, 157 (1994).
146. M.A. Moskowitz, Trends Pharmacol. Sci., 13, 307 (1992).
147. A. Orjaves-Venero and R. Rodes-Solanes, EP 628549-A1 (1994).
148. B.A. Whelan, I. Iriepa, E. Galvez, A. Orjales, A. Berisa, L. Labeage, A.G. Garcia, G. Uceda, J. Sanz-Aparicio and I. Fonseca, J. Pharm. Sci., 84, 101 (1995).
149. G.R.E. van Lommen, F.J. Fernandez-Gadea, J.I. Andres-Gil and M.E. Matesanz-Ballesteron, WO 9505381-A1 (1995).
150. T. Gungor, P. Malabre, J.M. Teulon, F. Camborde, J. Meignen, F. Hertz, A. Virone-Oddos, F. Caussade and A. Cloarec, J. Med. Chem., 37, 4307 (1994).

Chapter 3. Heterogeneity of Corticotropin Releasing Factor Receptors: Multiple Targets for the Treatment of CNS and Inflammatory Disorders

Errol B. De Souza, Timothy W. Lovenberg, Derek T. Chalmers, Dimitri E. Grigoriadis, Chen W. Liaw, Dominic P. Behan and James R. McCarthy
Neurocrine Biosciences, Inc., San Diego, CA 92121

Introduction - Corticotropin-releasing factor (CRF), a 41-residue peptide, plays a crucial role in integrating the body's overall response to stress. The CRF-adrenocorticotropin (ACTH)-glucocorticoid axis is central to the endocrine response to stress (1-5). In addition to its endocrine effects, immunohistochemical localization of CRF has demonstrated that the peptide has a broad extrahypothalamic distribution in the central nervous system (CNS) (6-8). CRF produces a wide spectrum of autonomic, electrophysiological and behavioral effects consistent with a neurotransmitter or neuromodulator role in the brain (2-5). Intracerebroventricular administration of CRF provokes stress-like responses including activation of the sympathetic nervous system (3,4,9), and inhibition of the parasympathetic nervous system (3,4,10) with consequential increases in plasma concentrations of epinephrine, norepinephrine and glucose; increases in heart rate and mean arterial blood pressure; inhibition of gastrointestinal functions including inhibition of gastric acid secretion. The behavioral profile following central adminstration of CRF is also characteristic of a compound which increases arousal and emotional reactivity to the environment. These effects of CRF include general arousal, as exhibited by increased locomotion, sniffing, grooming, and rearing in familiar surroundings and increased agitation in unfamiliar surroundings (3,4,11,12). In contrast, sexual receptivity (13) and feeding (14) are decreased. In addition to the neuromodulatory and neuroendocrine actions of CRF, recent evidence suggests that CRF may also play a significant role in integrating the response of the immune system to physiological, psychological and immunological stressors. In particular, a regulatory circuit interconnects the hypothalamic-pituitary-adrenal (HPA) axis with the immune system (5,15,16). CRF and immunostimulation induce, and glucocorticoids suppress pro-opiomelanocortin-derived peptide synthesis in leukocytes (15,16).

In this chapter, we describe some of our recent studies on a variety of CRF related targets including three functionally distinct receptor subtypes and the CRF-binding protein (CRF-BP). The characteristics of CRF receptors including their sequence homologies, pharmacological profiles and second messenger activities will be described. In addition, the differential localization of mRNA for CRF receptors and CRF-BP will be reviewed. Since clinical data suggest that CRF may be implicated in psychiatric, neurologic and immunological disorders (2,5,17,18), a brief description of the role of CRF, its receptors and its binding protein in the etiology and pathophysiology of these diseases will be included. Finally, an update on small molecule, non-peptide CRF receptor antagonists will be provided.

HETEROGENEITY OF CRF RECEPTORS

CRF_1 Receptor - The first cDNA encoding a CRF receptor has recently been identified using expression cloning from a human Cushing's corticotropic adenoma (19). This human cDNA encodes a 415-amino acid protein (designated as CRF_1 receptor) comprising seven putative membrane-spanning domains characteristic of G_s-coupled receptors (19). Species homologs for the CRF_1 receptor have also been isolated from rat brain (20,21) and mouse pituitary (22). In the human pituitary adenoma, an alternatively spliced form of the receptor

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

which includes an insert of 29 amino acids in the first intracellular loop has been reported, but species homologs of this transcript have yet to be identified (19). All forms of the CRF_1 receptor show homology (~ 30%) with receptors for growth hormone-releasing factor, vasoactive intestinal peptide (VIP), secretin, parathyroid hormone and calcitonin. There appear to be five potential N-glycosylation sites in the N-terminus regions (19) substantiating the glysosylation profiles determined in chemical affinity cross-linking studies (23). In addition, there are potential protein kinase C phosphorylation sites in the first and second intracellular loops and in the C-terminal tail, as well as casein kinase II and protein kinase A phosphorylation sites in the third intracellular loop (19). Tissue distribution of the CRF_1 receptor is consistent with the traditional radiolabeled CRF receptor with high abundance in the pituitary, cerebellum, and cerebral cortex (2,24).

Human or rat CRF_1 receptors expressed in stable cell lines (25) demonstrated reversible, saturable, high-affinity binding to CRF (K_d ~ 150 pM) and its related peptides with the pharmacological and functional characteristics comparable to those found in a variety of animal or human tissues (26,27). The pharmacological rank orders of potency in the stably transfected cell lines with either the human or rat CRF_1 receptors were identical to the established profile for the CRF receptor in the rat frontal cortex. The rank order of potencies for CRF-related and unrelated peptides were: sauvagine = urotensin I = ovine CRF (oCRF)= rat/human CRF (r/h CRF)= bovine CRF > d-Phe r/hCRF(12-41) > α-helical ovine CRF(9-41) >> r/hCRF(6-33), r/hCRF(9-33), r/hCRF(1-41)OH, VIP, arginine vasopressin. In addition, CRF_1 receptors transfected into these cell lines were coupled to a guanine nucleotide binding protein and when incubated in the presence of CRF, stimulated the production of cAMP from these cells with an EC_{50} of approximately 1 nM (25). Furthermore, the CRF stimulated cAMP production was competitively inhibited by selective peptide CRF receptor antagonists such as d-Phe CRF(12-41) or α-helical CRF(9-41) demonstrating, that the expressed CRF receptors in these cell lines were functional (25).

<u>CRF_2 Receptor</u> - Very recently, two rat brain cDNA clones were identified that encode two new members of the CRF receptor family, designated as the $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors (28). The $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors are produced from alternative splicing of a single gene which results in different N-terminal domains on the receptor proteins. These CRF_2 receptors encode proteins of 411 and 431 amino acids, respectively, each of which has ~70% identity to the known rat CRF_1 receptor over the entire coding region. When expressed in mouse Ltk$^-$ cells, the $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors stimulate cAMP production in a dose-dependent manner in response to CRF and the known non-mammalian CRF-related peptides sauvagine and urotensin I. The rank order of potency is different for both $CRF_{2\alpha}$ and $CRF_{2\beta}$ receptors, where $CRF_{2\alpha}$ exhibits the following profile: sauvagine > urotensin I ≥ r/hCRF > oCRF. $CRF_{2\beta}$ exhibits a pharmacological profile similar to CRF_1 with sauvagine being equipotent to urotensin and r/h CRF. Tissue distribution analysis by RNase protection assay shows differential distribution of CRF_2 receptors, in that $CRF_{2\alpha}$ is found primarily in the brain and $CRF_{2\beta}$ is expressed predominantly in the periphery in heart and skeletal muscle (29). Two other laboratories have recently cloned the mouse homolog of the $CRF_{2\beta}$ receptor and confirm its peripheral distribution and CRF_1 receptor-like pharmacology (30,31).

<u>CRF-Binding Protein</u> - Under normal conditions, the plasma levels of CRF remain low. However, CRF levels are markedly elevated in plasma during the late gestational stages of pregnancy (32-34). The source of the pregnancy-associated CRF is most likely the placenta since previous studies have demonstrated that the human placenta synthesizes CRF (35). The CRF in the maternal plasma is bioactive in releasing ACTH from cultured pituitary cells (33). In spite of the high levels of CRF in the maternal plasma, there is no evidence of markedly increased ACTH secretion or hypercortisolism in pregnant women (32). A plausible explanation for this paradoxical situation could be the presence of a binding protein in the plasma of pregnant women which modulates the biological actions of CRF (33,34). This

hypothesis was validated by the isolation of a CRF-binding protein (CRF-BP) from human plasma and its subsequent cloning and expression .

The CRF-BP was first isolated and purified to near homogeneity for sequencing and generation of oligonucleotide probes (36). Screening a human liver cDNA library using probes generated from the original amino acid sequence revealed a full length cDNA containing a 1.8 kb insert that encodes a novel protein of 322 amino acids (37). A single putative N-linked glycosylation site was found at amino acid 203 which agrees with the previous observation of the presence of asparagine-linked sugar moieties on the native protein (38). Subsequent screening of a rat cerebral cortical cDNA library, revealed the presence of a single clone containing a 1.85 kb insert predicting a protein of 322 amino acids which was 85% identical to the human CRF-BP (37). The putative glycosylation site seems to be conserved between the rat and human sequences (37). The CRF binding profile of these proteins appears to be similar with both the rat and human binding proteins having high affinity for r/hCRF ($K_d \approx 0.2$ nM) and very low affinity for oCRF ($K_i \approx 250$ nM). Furthermore, r/hCRF fragments that are inactive at CRF receptors [CRF(1-41)OH, CRF(6-33) and CRF(9-33)] have high affinity for the CRF-BP ($K_i \approx 0.2$ - 5 nM). Although there may be some similarities in the binding domains of the CRF-BP and the CRF receptors, these are distinct proteins each with unique characteristics and distributions.

Thus, there appears to be heterogeneity in CRF receptors and CRF-BP with respect to sequence, pharmacology and tissue distribution which would predict important functional differences between the CRF_1 receptor, CRF_2 receptor and CRF-BP. Table 1 below indicates the standardized nomenclature for the CRF receptor subtypes and indicates the alternate names for these subtypes that have appeared in the literature.

Table 1: Heterogeneity of CRF Receptors

Species	Receptor Subtype	Alternate Names	Length (aa)	Reference
Rat	CRF_1	CRF-RA	415	21
		PC-CRF		20
	$CRF_{2\alpha}$		411	28,29
	$CRF_{2\beta}$		431	28,29
	CRF-BP		322	37
Mouse	CRF_1		415	22
		PC-CRF		30
	$CRF_{2\beta}$	HM-CRF	431	30
		CRF-RB		31
Human	CRF_1	CRF-RA	415	19, 22
	CRF-BP		322	37

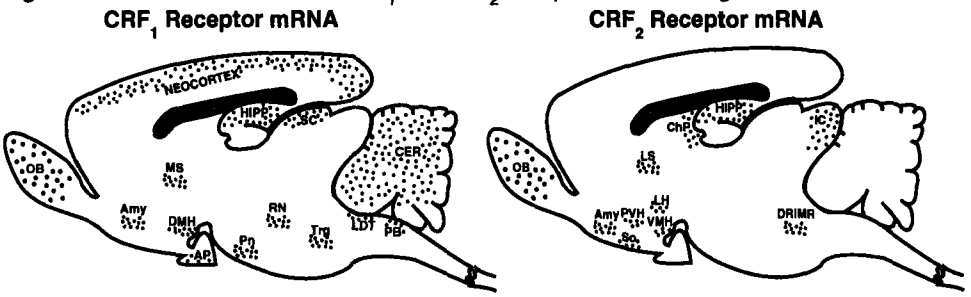
DISTRIBUTION OF CRF RECEPTORS AND CRF-BINDING PROTEIN

CRF Receptors - *In situ* hybridization histochemistry studies indicate heterogeneous anatomical distribution patterns for CRF_1 and CRF_2 receptor subtypes in brain (39). While CRF_1 receptor mRNA expression is most abundant in neocortical, cerebellar and sensory relay structures, CRF_2 receptor expression is generally localized to specific sub-cortical structures, most notably lateral septal nuclei and various hypothalamic areas (Figure 1). This heterogeneous distribution of CRF_1 and CRF_2 receptor mRNA suggests distinctive functional roles for each receptor in CRF-related systems. Thus, the selective expression of CRF_2 receptor mRNA within hypothalamic nuclei such as the ventromedial nucleus (VMH) and the paraventricular nucleus (PVH) indicates that the anxiogenic and anorexic actions of CRF in these nuclei are likely to be CRF_2 receptor-mediated. On the other hand, within the pituitary, CRF_1 receptor expression predominates over CRF_2 expression in both the

intermediate and anterior (AP) lobes indicating that CRF_1 receptors are primarily responsible for CRF-induced changes in ACTH release.

Examination of CRF_2 receptor splice variants, $CRF_{2\alpha}$ and $CRF_{2\beta}$, indicates distinctive anatomical distributions: The $CRF_{2\alpha}$ form being primarily expressed within the brain and the $CRF_{2\beta}$ variant being found in both the CNS and periphery (29). Within the brain, it appears that the $CRF_{2\alpha}$ form represents the predominant neuronal CRF_2 variant while the $CRF_{2\beta}$ splice form is localized on non-neuronal elements, the choroid plexus and cerebral blood vessels. In all these brain areas, $CRF_{2\alpha}$ mRNA is present only in low densities. In peripheral organs, $CRF_{2\beta}$ mRNA is expressed at high levels in both cardiac and skeletal muscle with lower levels evident in both lung and intestine. This heterogeneous distribution of CRF_2 receptor splice variants and their specific pharmacological profiles provides the potential opportunity for tissue-specific drug design.

Figure 1. *Schematic Illustration of CRF_1 and CRF_2 Receptor mRNA in Sagittal Section*



Key: • = receptor mRNA expression

Abbreviations: OB, olfactory bulb; Amy, amygdala; MS, medial septum; DMH, dorsomedial hypothalamic nucleus; AP, anterior pituitary; HIPP, hippocampus; SC, superior colliculus; Pn, pontine nuclei; RN, red nuclei; Trg, trigeminal nuclei; LDT, lateral dorsal tegmental nuclei; PB, parabrachial nuclei; CER, cerebellum; LS, lateral septum; PVH, paraventricular nuclei; So, supraoptic nuclei; LH, lateral hypothalamus; VMH, ventromedial hypothalamic nucleus; ChP, choroid plexus; IC, inferior colliculus; DR/MR, dorsal, medial raphe.

CRF-Binding Protein - Although the human and rat forms of the CRF-BP are homologous, there is a somewhat different anatomical distribution pattern in the two species. The human form of the binding protein has been found abundantly in tissues such as liver, placenta and brain while in the rat, levels of mRNA for the binding protein have only been localized in the brain and pituitary (37). Peripheral expression of the binding protein may have its greatest utility in the modulation and control of the elevated circulating levels of CRF induced by various normal physiological conditions (see above). In addition, expression of this binding protein in the brain and pituitary offers additional mechanisms by which CRF-related neuronal or neuroendocrine actions may be modulated.

CRF-BP has been localized to a variety of brain regions including neocortex, hippocampus (primarily in the dentate gyrus) and olfactory bulb. In the basal forebrain, mRNA is localized to the amygdaloid complex with a distinct lack of immunostained cells in the medial nucleus. CRF-BP immunoreactivity is also present in the brain stem particularly in the auditory, vestibular and trigeminal systems, raphe nuclei of the midbrain and pons and the reticular formation (40). In addition, high expression levels of binding protein mRNA are seen in the anterior pituitary, predominantly restricted to the corticotrope cells. Expression of this protein in the corticotropes strongly suggests that the CRF-BP is involved in the regulation of neuroendocrine functions of CRF by limiting and/or affecting the interactions of CRF with its receptor which is also known to reside on corticotropes. The detailed role of the binding protein however in regulating pituitary-adrenal function remains to be elucidated.

ROLE OF CRF IN CNS DISORDERS

Major Depression and Anxiety Disorders - Many patients with major depression are hypercortisolemic and exhibit an abnormal dexamethasone suppression test. Given the primary role of CRF in stimulating pituitary-adrenocortical secretion, the hypothesis has been put forth that hypersecretion/hyperactivity of CRF in brain might underlie the hypercortisolemia and symptomatology seen in major depression. The concentration of CRF is significantly increased in the cerebrospinal fluid (CSF) of drug-free individuals (4,18,41), and a significant positive correlation is observed between CRF concentrations in the CSF and the degree of post-dexamethasone suppression of plasma cortisol (42). Furthermore, the observation of a decrease in CRF binding sites in the frontal cerebral cortex of suicide victims compared to controls is consistent with the hypothesis that CRF is hypersecreted in major depression (43). The increased CSF concentrations of CRF seen in depressed individuals are decreased following treatment with electroconvulsive therapy (44). In addition, a blunted ACTH response to intravenously administered ovine or human CRF is observed in depressed patients when compared to normal controls (45,46). The blunted ACTH response to exogenous CRF seen in depressed patients may be due to the intact negative feedback of cortisol on the corticotrophs, due to a compensatory decrease in CRF receptors subsequent to chronic hypersecretion of the peptide and/or desensitization of the pituitary corticotrophs that respond to CRF.

A number of studies suggest that anxiety-related disorders (such as panic disorder and generalized anxiety disorder) and depression are independent syndromes which share both clinical and biological characteristics. The potent anxiogenic effects of CRF in rodents and primates (12) have led to the suggestion that CRF may also be involved in anxiety-related disorders. A role for CRF in panic disorder has been suggested by observations of blunted ACTH responses to intravenously administered CRF in panic disorder patients when compared to controls (47). The blunted ACTH response to CRF in panic disorder patients most likely reflects a process occurring at or above the hypothalamus, resulting in excess secretion of endogenous CRF.

Anorexia Nervosa - Anorexia nervosa is an eating disorder characterized by the tremendous weight loss in the pursuit of thinness. There is similar pathophysiology in anorexia nervosa and in depression including the manifestation of hypercortisolism, hypothalamic hypogonadism and anorexia. Furthermore, the incidence of depression in anorexia nervosa patients is high. Like depressed patients, anorexics show a markedly attenuated ACTH response to intravenously administered CRF (4,18,46). When underweight anorexic subjects are studied after their body weight had been restored to normal, their basal hypercortisolism, increased levels of CRF in the CSF, and diminished ACTH response to exogenous CRF all return to normal at varying periods during the recovery phase (4,18,46). CRF can potently inhibit food consumption in rats (14) which further suggests that the hypersecretion of CRF may be responsible for the weight loss observed in anorexics. In addition, the observation that central administration of CRF diminishes a variety of reproductive functions (48) lends relevance to the clinical observations of hypogonadism in anorexics.

Alzheimer's Disease - Several studies have provided evidence in support of alterations in CRF in Alzheimer's disease (AD). There are decreases in CRF content (49-52), reciprocal increases in CRF receptors (50,51) and no significant alterations in CRF-BP (53) in cerebral cortical areas affected in AD such as the temporal, parietal and occipital cortices. The reductions in CRF and increases in CRF receptors are all greater than 50% of the corresponding control values. Chemical cross-linking studies have demonstrated a normal pattern of labeling of cerebral cortical CRF receptors in AD when compared to age-matched controls (54). The up-regulation in cerebral cortical CRF receptors in AD under conditions in which the endogenous peptide is reduced suggests that CRF-receptive cells may be preserved in the cortex in AD. The reduction in cortical CRF content may be due to selective degeneration of CRF neurons intrinsic to the cerebral cortex or it could be due to dysfunction

of CRF neurons innervating the cortex from other brain areas. Additional evidence for a role for CRF in AD is provided by observations of decreases in CRF in other brain areas including the caudate (49) and decreased concentrations of CRF in the CSF (55,56). Furthermore, a significant correlation is evident between CSF CRF and the global neuropsychological impairment ratings suggesting that greater cognitive impairment is associated with lower CSF concentrations of CRF (57).

Immunocytochemical observations demonstrating morphological alterations in CRF neurons in AD complement the studies described above. In AD, swollen, tortuous CRF-immunostained axons, termed fiber abnormalities, are clearly distinguishable from the surrounding normal neurons and are also seen in conjunction with amyloid deposits associated with senile plaques (58). Furthermore, the total number of CRF-immunostained axons is reduced in the amygdala of Alzheimer's patients (58). Interestingly, the expression of CRF antigen in neurons is not globally reduced in Alzheimer's patients. CRF immunostaining of perikarya and axons located in the hypothalamic paraventricular nucleus is much more intense in AD than in controls (58). Increased immunostaining of the paraventricular neurons in AD, if truly representative of increased content of CRF, could be related to increased amounts of CRF mRNA in these cells or increased translation of available mRNA. The increased expression and/or release of CRF from the paraventricular nucleus of the hypothalamus would provide a reasonable explanation for the hypercortisolemia and decreased ACTH response to exogenous CRF often seen in Alzheimer's patients (59,60).

Other Neurological Disorders - Alterations in brain concentrations of CRF have been reported in other neurological diseases. For example, in cases of Parkinson's disease with dementia that also show pathological features of AD, CRF content is decreased and shows a pattern similar to those cases exhibiting the pathology of AD alone (17,51,52). Specimens from patients with Parkinson's disease who did not have the histopathology characteristic of AD also demonstrate reductions of CRF content, although the reductions are less marked than in cases of combined AD and Parkinson's disease. Normal levels of CRF have been reported in the hypothalamus in Parkinson's disease (61), suggesting that the loss of CRF in the cerebral cortex is not generalized. CRF is deceased to ~ 50% of the control values in the frontal, temporal and occipital lobes of patients with progressive supranuclear palsy (17,52), a rare neurodegenerative disorder that shares certain clinical and pathological features with AD.

The similarity of the changes in CRF found in the context of the three neurological diseases associated with Alzheimer-type pathology raised the possibility that cerebral cortical reduction is nothing more than a nonspecific sequela of the disease process. In Huntington's disease, a neurological disorder in which minimal cerebral cortical pathology is present, the CRF content in the frontal, temporal, parietal, occipital and cingulate cortices and in the globus pallidus is not significantly different from that seen in neurologically normal controls (51,62). However, the CRF content in the caudate nucleus and putamen of the basal ganglia (a brain area that is severely affected in the disease) is less than 40% of the CRF concentrations seen in controls (62). The localization of the CRF changes to only affected brain regions in the four neurodegenerative disorders described suggest that CRF has an important role in the pathology of these dementias.

ROLE OF CRF IN INFLAMMATORY DISORDERS

Recent data provide evidence for a direct pro-inflammatory action of CRF in rat models of inflammation and arthritis. Carrageenin, a seaweed polysaccharide, elicits a chemical inflammatory response in rats. In this acute model of inflammation, increased levels of immunoreactive CRF were detected in the inflamed area but not in the systemic circulation (63). Furthermore, immunoneutralization of CRF reduced both the volume and cellularity of the exudate in the carrageenin model indicating that CRF has pro-inflammatory actions (63). The anti-inflammatory effect of the anti-CRF antibody was comparable to the anti-inflammatory effects of anti-tumor necrosis factor-a-antibody (63). Recent studies in

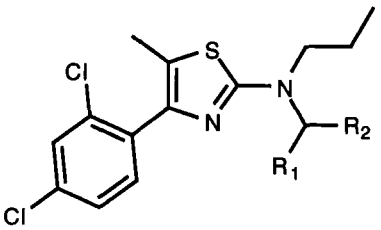
experimental rat models of arthritis further substantiate the proinflammatory paracrine or autocrine effects of peripheral CRF. CRF expression is markedly increased in the joints and surrounding tissues of arthritis-susceptible LEW/N rats with streptococcal cell wall (SCW)- and adjuvant-induced arthritis, while it is not increased in similarly treated F344/N arthritis-resistant rats, and is only transiently increased in congenitally athymic nude LEW.rnu/rnu rats (64). CRF mRNA and CRF receptors were present in inflamed synovia of LEW/N rats and increases in CRF markers paralleled increases in other pro-inflammatory peptides such as substance P (64). A recent clinical study examined synovial fluids and tissues from patients with rheumatoid arthritis (RA) or osteroarthritis (OA) and normal individuals in order to determine the role of CRF in human inflammatory arthritis (65). There was a marked enhanced expression of immunoreactive CRF *in situ* in synovium from patients, which was significantly greater in RA than in OA; the extent and intensity of immunostaining correlated significantly with the intensity of mononuclear cell infiltration (65). Furthermore, the concentrations of CRF were approximately six-fold higher in RA than OA synovial fluids (65). Overall, these data substantiate an important autocrine/paracrine pro-inflammatory role for CRF at the inflammatory site in arthritis and suggest that therapies directed at inactivation of CRF or blocking the effects of CRF may represent novel anti-inflammatory agents.

THERAPEUTIC STRATEGIES

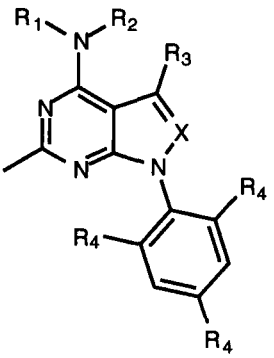
Diseases Associated with High Levels of CRF - Strategies to counter the deleterious effects associated with high levels of CRF include inhibition of CRF synthesis and secretion, inactivation of CRF (either through increased metabolism or neutalization) or antagonism of the effects of CRF through receptor blockade. Many of the current therapies used for the treament of depression and anxiety-related disorders may, in fact, produce their therapeutic effects through modulation of CRF synthesis and release. For example, benzodiazepines are potent inhibitors of CRF secretion both *in vitro* (66) and *in vivo* (67) and appear to counteract the effects of stress (67). Furthermore, chronic benzodiazepine treatment results in upregulation of CRF receptors in select brain areas in keeping with inhibition of CRF release (68). Furthermore, behavioral studies in rodents demonstrate interactions between benzodiazepine anxiolytics and CRF. Chlorodiazepoxide attenuates the anxiogenic effects of CRF in both the conflict test (69,70) and in the acoustic startle test (71). Similar behavioral interactions have also been noted with $5HT_{1A}$ class of anxiolytics and CRF (72,73). More recently, it has been demonstrated that chronic treatment of rats with diverse classes of antidepressants such as tricyclics (amitriptyline) or monoamine oxidase-A inhibitor (moclebemide) attenuates CRF activity (74,75).

Anti-CRF antibodies or CRF-BP may be useful for treatment of diseases associated with elevated peripheral concentrations of CRF such as rheumatoid arthritis. This strategy is however limited to peripheral disorders and has the disadvantages associated with recombinant protein or antibody therapies that are not amenable to oral administration. The most direct way to counter the elevated concentrations of CRF is to develop receptor antagonists. While stable peptide antagonists may be considered, access to the brain following systemic administration and oral bioavailability with such compounds limits this approach. Recently, several classes of non-peptide CRF receptor antagonist leads have been identified. An update of some of these non-peptide CRF receptor antagonists follows.

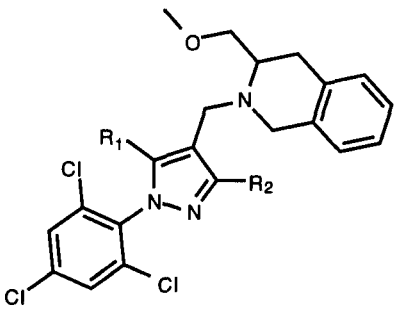
Non-Peptide CRF Receptor Antagonists - Several classes of non-peptide CRF receptor antagonist leads have been reported in the past year that have been identified through use of high throughput screening of chemical libraries and structural modification. Thiazole analogs of **1** inhibit binding of [^{125}I]-CRF to CRF receptors on rat cortex membranes (76). No K_i values or biological data were published in the patent application. Four world patent applications reported compounds represented by **2** - **6** as CRF receptor antagonists (77-80). These compounds are claimed to inhibit binding of [^{125}I]-CRF to its receptor in the low nanomolar range.



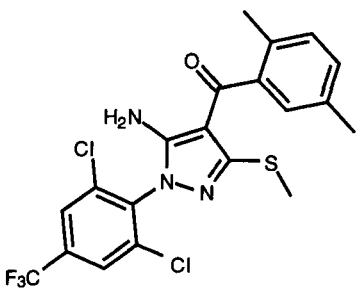
1 :R_1, R_2 = aryl, pyridyl, Me Et, cyclopropyl, cyclobutyl cyclopentyl, imidazoyl



2: R_1 = n-Bu, R_2 = Et R_3 = R_4 = Me, X = C-Me
3: R_1 = R_2 = n-Pr R_3 = SMe, R_4 = Cl, X = N



4: R_1 = NMe_2, R_2 = SMe
5: R_1 = R_2 = Et



6

Diseases Associated with Low Levels of CRF - In view of the noted deficits in CRF and cognition in AD, it would appear that the CRF system may offer hope for potential drug therapy for disorders of memory associated with Alzheimer's-type and related dementias. CRF administration in rodents increases learning and memory in a variety of behavioral paradigms, further substantiating its utility in counteracting memory deficits (81,82). While postsynaptic CRF receptor agonist-related therapy can be considered for dementia, the potential for anxiogenic "stress-like" side effects of a CRF agonist must also be considered since the receptor is widely distributed in brain areas involved in both memory processing as well as in limbic and brain stem areas involved with arousal and emotional responses (24). In fact, doses of CRF that increase learning and memory (81,82) also show increases in behavioral responses to stressors in rodents (12). The identification of a membrane-associated form of CRF-BP with high affinity for CRF in brain and the ability to inactivate CRF (83) provides a novel target to modulate endogenous levels of CRF in select brain areas enriched in the binding protein. The differential distribution of the CRF-BP (40) and CRF receptors (24) in rat brain with an enrichment of the CRF-BP in brain areas (e. g. cerebral cortex and hippocampus) that are affected in AD and are involved in learning and memory processes (40) provide multiple distinct sites of interaction with CRF for potential exploitation in the treatment of central defects in CRF neurotransmitter function. Selective ligands that dissociate CRF from the CRF/CRF-BP complex and thereby increase synaptic levels of "free CRF" may have wide applicability for the treatment of disorders/conditions associated with low levels of CRF in brain.

SUMMARY AND FUTURE DIRECTIONS

The actions of CRF in the brain and in the periphery are mediated through multiple binding sites. There is a heterogeneity of CRF binding sites with respect to sequence, pharmacology and tissue distribution. There are three receptors, CRF_1, $CRF2\alpha$ and $CRF2\beta$, which encode 411, 415 and 431 amino acid proteins, respectively comprising seven putative membrane-spanning domains characteristic of G_S-coupled receptors. All three receptors transduce a signal which involved stimulation of cAMP production. CRF receptor antagonists may represent novel agents for treatment of disorders associated with elevated levels of CRF. The recent identification of high-affinity non-peptide CRF receptor antagonists should allow for rapid progress in drug development of CRF receptor antagonists. In addition to the receptors, the actions of CRF in brain and in the periphery can also be modulated by a binding protein of 322 amino acids. While the precise function of the CRF-BP remains to be determined, it appears to play a role in binding and functionally inactivating CRF and CRF-BP ligand inhibitors may represent a potential mechanism for increasing endogenous CRF levels .

References

1. W. Vale, J. Spiess, C. Rivier and J. Rivier, Science, 213, 1394 (1981).
2. E.B. De Souza and C.B. Nemeroff (eds.), "Corticotropin-Releasing Factor: Basic and Clinical Studies of a Neuropeptide", CRC Press, Boca Raton, 1990.
3. A.J. Dunn and C.W. Berridge, Brain Res. Rev., 15, 71 (1990).
4. M.J. Owens and C.B. Nemeroff, Pharmacol. Rev., 43, 425 (1991).
5. E.B. De Souza and D.E. Grigoriadis, in "Psychopharmacology: The Fourth Generation of Progress" (F.E. Bloom and D.J. Kupfer, eds.), p. 505, Raven Press, New York, 1995.
6. S. Cummings, R. Elde, J. Ells and A. Lindvall, J. Neurosci., 3, 1355 (1983).
7. L.W. Swanson, P.E. Sawchenko, J. Rivier and W. Vale, Neuroendocrinology, 36, 165 (1983).
8. P.E. Sawchenko, T. Imaki, E. Potter, K. Kovacs, J. Imaki and W. Vale, Ciba Found. Symp. 172, 5 (1993)..
9. M.R. Brown and L.A. Fisher, in "Corticotropin-Releasing Factor: Basic and Clinical Studies of a Neuropeptide" (E.B. De Souza and C.B. Nemeroff, eds.), p. 291. CRC Press, Boca Raton, 1990.
10. Y. Tache, H. Monnikes, B. Bonaz and J. Rivier, Ann. N.Y. Acad. Sci., 697, 233 (1993).
11. N.H. Kalin, in "Corticotropin-Releasing Factor: Basic and Clinical Studies of a Neuropeptide" (E.B. De Souza and C.B. Nemeroff, eds.), p. 275. CRC Press, Boca Raton, 1990.
12. G.F. Koob, S.C. Heinrichs, E. Merlo Pich, F. Menzaghi, H. Baldwin, K. Miczek and K.T. Britton, Ciba Foundation Symp. 172, 277 (1993).
13. D.J.S. Sirinathsinghji, L.H. Rees, J. Rivier and W. Vale, Nature, 305, 232 (1983).
14. J. Morley and A.S. Levine, in "Corticotropin-Releasing Factor: Basic and Clinical Studies of a Neuropeptide" (E.B. De Souza and C.B. Nemeroff, eds.), p. 267. CRC Press, Boca Raton, 1990.
15. J.E. Blalock, Physiol. Rev., 69, 1 (1989).
16. E..L. Webster, D.E. Grigoriadis and E.B. De Souza, in "Stress, Neuropeptides and Systemic Disease" (J.A. McCubbin, P.G. Kaufmann and C.B. Nemeroff, eds.), p. 233. Academic Press, San Diego, 1991.
17. E.B. De Souza, Hospital Practice, 23, 59 (1988).
18. E.B. De Souza, Ann. Rep. Med. Chem. 25, 215 (1990).
19. R. Chen, K.A. Lewis, M.H. Perrin and W.W. Vale, Proc. Natl. Acad. Sci. USA, 90, 8967 (1993).
20. C-P. Chang, R.V. Pearse II, S. O'Connell and M.G. Rosenfeld, Neuron 11, 1187 (1993).
21. M.H. Perrin, C.J. Donaldson, R. Chen, K.A. Lewis and W.W. Vale, Endocrinology, 133, 3058 (1993).
22. N. Vita, P. Laurent, S. Lefort, P. Chalon, J.M. Lelais, M. Kaghad, G. Le Fur, D. Caput and P. Ferrara, FEBS Lett., 335, 1 (1993).
23. D.E. Grigoriadis and E.B. De Souza, Endocrinology, 125, 1877 (1989).
24. E.B. De Souza, T.R. Insel, M.H. Perrin, J. Rivier, W. Vale and M.J. Kuhar, J. Neurosci. 5, 3183 (1985).
25. D.E. Grigoriadis, C. Liaw, T. Oltersdorf and E.B. De Souza, Soc. Neurosci. Abstr. 20, 552.7 (1994).
26. E.B. De Souza, J. Neurosci., 7, 88 (1987).
27. E.B. De Souza, in "Handbook of Chemical Neuroanatomy - Neuropeptide Receptors in the CNS, Part III" (A. Bjorklund, T. Hokfelt and M.J. Kuhar, eds.), p. 145. Elsevier, Amsterdam, 1992.
28. T.W. Lovenberg, C.W. Liaw, D.E. Grigoriadis, W. Clevenger, D.T. Chalmers, E.B. De Souza and T. Oltersdorf, Proc. Natl. Acad. Sci. USA, 92, 836 (1995).
29. T.W. Lovenberg, D.T. Chalmers, C. Liu and E.B. De Souza, Endocrinology, (submitted).
30. T. Kishimoto, R.V. Pearse II, C.R. Lin and M.G. Rosenfeld, Proc. Natl. Acad. Sci. USA,, 92, 1108 (1995).
31. M. Perrin, C. Donaldson, R. Chen, A. Blount, T. Berggren, L. Bilezikjian, P. Sawchenko and W.W. Vale, Proc. Natl. Acad. Sci. USA,, 92, 2969 (1995).
32. T. Laatikainen, T. Virtanen, I. Raisanen and K. Salminen, Neuropeptides, 10, 343 (1987).
33. E.A. Linton, C.D.A. Wolfe, D.P. Behan and P.J. Lowry, Clin. Endocrinol., 28, 315 (1988).

34. T. Suda, M. Iwashita, F. Tozawa , T. Ushiyama, N. Tamori, T. Sumitomo, Y. Nakagami, H. Demura and K. Shizume, J. Clin. Endocrinol. Metab., 67, 1278 (1988).
35. T. Shibasaki, E. Odagiri, K. Shizume and N. Ling, J. Clin. Endocrinol. Metab., 55, 384 (1982).
36. D.P. Behan, E.A. Linton and P.J. Lowry, J. Endocrinol., 122, 23 (1989).
37. E. Potter, D. Behan, W.H. Fisher, E.A. Linton, P.J. Lowry and W.W. Vale, Nature, 349, 423 (1991).
38. T. Suda, T. Sumitomo, F. Tozawa, T. Ushiyama and H. Demura, Biochem. Biophys. Res. Commun., 165, 703 (1989).
39. D.T. Chalmers, T.W. Lovenberg and E.B. De Souza, J. Neurosci., (submitted).
40. E. Potter, D.P. Behan, E.A. Linton, P.J. Lowry, P.E. Sawchenko and W.W. Vale, Proc. Natl. Acad. Sci. USA, 89, 4192 (1992).
41. C.B. Nemeroff, E. Widerlov, G. Bissette, H. Walleus, I. Karlsson, K. Eklund, C.D. Kilts, P.T. Loosen and W. Vale, Science, 226, 1342 (1984).
42. A. Roy, D. Pickar, S. Paul, A. Andorn, G.P. Chrousos and P.W. Gold, Am. J. Psychiatry, 144, 641 (1987).
43. C. Nemeroff, M. Owens, G. Bissette, A.. Andorn and M. Stanley, Arch. Gen. Psychiatry, 45, 577 (1988).
44. C.B. Nemeroff, G. Bissette, H. Akil and M. Fink, Br. J. Psychiatr., 158, 59 (1991).
45. F. Holsboer, O.A. Muller, H.G. Doerr, W.G. Sippell, A. Gerken, A. Steiger, E. Boll and O. Benker, Psychoneuroendocrinology, 9, 147 (1984).
46. P.W. Gold, D.L. Loriaux, A. Roy, M.A. Kling, J.R. Calabrese, C.H. Kellner, L.K. Nieman, R.M. Post, D. Pickar, W. Gallucci, P. Avgerinos, S. Paul, E. Oldfield, G.B. Cutler, Jr., and G.P. Chrousos, New Engl. J. Med., 314, 1129 (1986).
47. P. Roy-Byrne, T. Uhde, R. Post, W. Galluci, G. Chrousos and P. Gold, Am. J. Psych. 143, 896 (1986).
48. C. Rivier and W. Vale, J. Clin. Invest. 75, 689 (1984).
49. G. Bissette, G. Reynolds, C. Kilts, W. Widerlov and C. Nemeroff, J. Am. Med. Assoc., 254, 3067 (1985).
50. E.B. De Souza, P.J. Whitehouse, M.J. Kuhar, D.L. Price and W.W. Vale, Nature, 319, 593 (1986).
51. E.B. De Souza, P.J. Whitehouse, M. Kuhar, D. Price and W. Vale, Ann. N.Y. Acad. Sci., 512, 237 (1987).
52. P.J. Whitehouse, W.W. Vale, R.M. Zweig, H.S. Singer, R. Mayeux, D.L. Price and E.B. De Souza, Neurology, 37, 905 (1987).
53. D.P. Behan, J. Troncoso, N. Ling and E.B. De Souza, Soc. Neurosci. Abstr. 20, 552.10 (1994).
54. D.E. Grigoriadis, R.G. Struble, D.L. Price and E.B. De Souza, Neuropharmacology, 28, 761 (1989).
55. M.M. Mouradian, J.M. Farah, Jr., E. Mohr, G. Fabbrini, T.L. O'Donohue and T.N. Chase, Neuropeptides, 8, 393 (1986).
56. C. May, S.I. Rapoport, T.P. Tomai, G.P. Chrousos and P.W. Gold, Neurology, 37, 535 (1987).
57. N. Pomara, R.R. Singh, D. Deptula, P.A. LeWitt, G. Bissette, M. Stanley and C.B. Nemeroff, Biol. Psychiatry 26, 500 (1989).
58. R.E. Powers, L.C. Walker, E.B. De Souza, W.W. Vale, R.G. Struble, P.J. Whitehouse and D.L. Price, Synapse, 1, 405 (1987).
59. K.P. Lesch, R. Ihl, L. Frohlich, R. Rupprecht, U. Muller, H.M. Schulte and K. Maurer, Psychiatry Res. 33, 107 (1990).
60. C. Dodt, J. Dittmann, J. Hruby, E. Spath-Schwalbe, J. Born, R. Schuttler and H.L. Fehm, J. Clin. Endocrinol. Metab. 72, 272 (1991).
61. B. Conte-Devolx, M. Grino, A. Nieoullon, E. Javoy-Agid, E. Castanas, V. Guillaume, M.C. Tonon, H. Vaudry and C. Oliver, Neurosci. Lett., 56, 217 (1985).
62. E.B. De Souza, P.J. Whitehouse, S.E. Folstein, D.L. Price and W.W. Vale, Brain Res. 437, 355 (1987).
63. K. Karalis, H. Sano, J. Redwine, S. Listwak, R.L. Wilder and G.P. Chrousos, Science, 254, 421 (1991).
64. L.J. Crofford, H. Sano, K. Karalis, E.L. Webster, E.A. Goldmuntz, G.P. Chrousos and R.L. Wilder, J. Clin. Invest., 90, 2555 (1992).
65. L.J. Crofford, H. Sano, K. Karalis, T.C. Friedman, H.R. Epps, E.F. Remmers, P. Mathern, G.P. Chrousos and R.L. Wilder, J. Immunol., 151, 1587 (1993).
66. A.E. Calogero, W.T. Gallucci, G.P. Chrousos and P.W. Gold, Brain Res., 463, 23 (1988).
67. M.J. Owens, G. Bissette and C.B. Nemeroff, Synapse, 4, 196 (1989).
68. D.E. Grigoriadis, D. Pearsall and E.B. De Souza, Neuropsychopharmacology, 2, 53 (1989).
69. K.T. Britton, J. Morgan, J. Rivier, W. Vale and G.F. Koob, Psychopharmcology, 86, 170 (1985).
70. K.T. Britton, G. Lee and G.F. Koob, Psychopharmacology, 94, 306 (1988).
71. N.R. Swerdlow, M.A. Geyer, W.W. Vale and G.F. Koob, Psychopharmacology, 88, 147 (1986).
72. A.J. Lazosky and D.L. Britton, Psychopharmacology, 104, 132 (1991).
73. J.A. Martinez and L. Bueno, Eur. J. Pharmacol., 202, 379 (1991).
74. J.M.H.M. Reul, I. Stec, M. Soder and F. Holsboer, Endocrinology, 133, 312 (1993).
75. J. Reul, M. Labeur, D. Grigoriadis, E.. De Souza and F. Holsboer, Neuroendocrinol., 60, 509 (1994).
76. C. Gautier, D. Gully, P. Roger, G. Valette and C.G. Wermuth, Eur. Patent Appl., EP 0 576 350 A1 (1993).
77. W.S. Faraci and W.M. Welch, Jr., World Patent Application, WO 94/13643 (1994).
78. G.M. Bright, World Patentr Application, WO 94/13644 (1994).
79. G.M. Bright and W.M. Welch, Jr., World Patent Application, WO 94/13661 (1994).
80. Y.L. Chen, World Patent Application, WO 94/13677 (1994).
81. G.F. Koob and F.E. Bloom, Fed. Proc., 44, 259 (1985).
82. K.C. Liang and E.H.Y. Lee, Psychopharmacology 96, 232 (1988).
83. D.P. Behan, E. Potter, S. Sutton, W. Fischer, P. Lowry and W. Vale, Ann. N.Y. Acad. Sci., 697, 1 (1993).

Chapter 4. Emerging Opportunities in Neuroinflammatory Mechanisms of Neurodegeneration

Kazumi Shiosaki and Pamela Puttfarcken
Neuroscience Research, Abbott Laboratories, Abbott Park, IL 60064

Introduction - The brain has been historically considered an immunologically privileged organ. This perception was due in part to the presence of the blood-brain barrier (BBB) that prevents entry into the brain of most blood-borne cells, proteins and other factors that are involved in regulation of the peripheral immune system (1). However, mounting evidence indicates that this privilege is not absolute and that a number of immunological mechanisms can actively operate within the central nervous system (CNS), particularly in response to disease or injury. These findings have spawned the rapidly evolving field of neuroimmunology, and with it the emergence of opportunities to develop novel therapeutic agents for neurodegenerative diseases.

IMMUNE FUNCTION IN THE CNS

Under normal circumstances, the brain can be considered immunologically quiescent. However when affected by various diseases or subjected to insult, the brain can become highly immunocompetent. A number of studies using sensitive and selective probes have found significant increases of immune function markers in diseased or injured brains relative to normal controls (2). The localization of these markers was often associated directly with the pathology inherent in the disease or insult (3). Microglial cells, activated in many chronic neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), AIDS-related dementia, multiple sclerosis (MS) as well as in ischemic damage resulting from stroke or head injury, are developmentally related to blood monocytes and tissue macrophages, and function in a similar capacity as their peripheral counterparts in being responsible for scavenging cellular debris (4 - 6). Infiltration of peripherally-derived immune cells into the brain can occur under certain conditions, particularly in disease states and after injuries that compromise the BBB (7). In addition, various brain-derived cell culture systems contain and express numerous immunological receptors and factors that can be upregulated when subjected to chemical or physical insult. Together, the evidence strongly supports an immune response to insult in the CNS, and this chapter will focus on a selected group of factors that have specific associations with various neurodegenerative diseases.

Cytokines - Cytokines comprise an expanding family of polypeptides that exert a broad range of biological activities ranging from normal development and maintenance of cells to initiation of an inflammatory response to disease or external challenge. Overproduction and overactivity of cytokines are often associated with pathological situations. Currently eight different cytokine families have been identified: interferons (INF), interleukins (IL), tumor necrosis factors (TNF), chemokines, colony stimulating factors, growth factors, neurotrophins and neuropoietins. Of these cytokines, the INFs, ILs and TNFs appear to be consistently and significantly unregulated in various neurodegenerative diseases, including AD, MS and AIDS dementia. Comprehensive reviews on cytokines and their actions in the CNS have appeared (8 - 12).

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

The family of INFs include more than 20 different proteins that can be classified into four major groups: α, β, ω (type 1), and γ (type 2). INFs bind to specific membrane-bound receptors that are characterized according to their recognition of either the type 1 or type 2 class of INFs. Type 1 INF receptors are found on all cells, whereas type 2 receptors are located on T lymphocytes and natural killer cells. The INF type 1 receptor is associated with activation of tyrosine and janus kinase (JAK) 1, and stimulation of INF type 2 receptor activates both JAK 1 and JAK 2 (13). Activation of these kinases leads to phosphorylation of various cytoplasmic proteins involved in signal transduction. Regions of the receptors responsible for binding INFs and eliciting function have been identified through site-directed mutagenesis. A comprehensive review on INF proteins and receptors has been published (13).

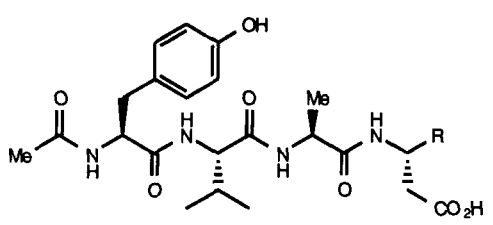
The biological effects of INFs include antiviral, antiproliferative, antitumor, immunomodulatory, and hormonal actions (14). In the CNS, type 1 INFs, e.g. INF-α, inhibit MHC class I expression, increase the cytotoxic activity of CD8 T-cells, and inhibit astrocyte proliferation. The type 2 INF-γ can modify the immune status in the CNS by inducing MHC antigen expression on astrocytes and microglia, and stimulating complement gene expression. Although glial cells appear to be the main source of INF in the brain, immunoreactivity for INF-γ has been localized to a subpopulation of neuronal cell bodies and nerve terminals in both the central and peripheral nervous systems (15). *In vitro* studies indicate that INF-α inhibits astrocyte proliferation and exerts modulatory effects on neuronal activity, including stimulation of neuronal choline acetyltransferase and altering firing rate of cells in rat brain (16, 17). INF-γ induces maturation of both astrocytes and mouse neurons in culture (18) while treatment of human astrocytes with INF-γ induces expression of HLA-DR surface antigens (19).

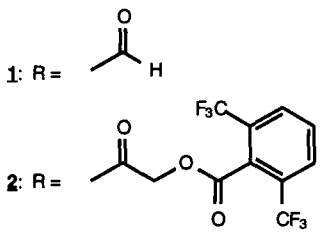
The IL gene family consists of glycosylated or phosphorylated proteins that include IL-1α and β, the endogenous IL-1 receptor antagonist (IL-1ra) and IL-6 (11). IL-1α and β, along with TNF-α and IL-6, are considered major mediators in the pathogenesis of acute and chronic inflammatory diseases. Two IL-1 receptors designated as type I and type II have been identified in the periphery and have been reviewed recently (11, 12). The IL-1 type I receptor binds both IL-1α and IL-1β with nearly equal affinities and is responsible for eliciting the spectrum of peripheral effects associated with IL-1. A signaling function for the IL-1 type II receptor has not been defined, and it may serve as a clearance receptor (20). The biologically active IL-1 receptors in the CNS and their cellular localizations are not known. IL-1 binding sites are found throughout the brain and are upregulated after injury (11), and mRNA for IL-1 type I receptors has been identified in CNS tissue, although its cellular distribution has not been established (21). One study failed to detect mRNA for the type II receptor in the brain (21). However, central administration of an antibody raised against the IL-1 type II receptor blocked effects of centrally administered IL-1, suggesting the presence and function for this receptor subtype in the brain (22). Whether this receptor is distinct or similar to the peripheral type II receptor is not known. Additional IL-1 receptors in the CNS have been proposed based on the inability to block some actions of IL-1 with IL-1ra (9, 10).

IL-1 exerts diverse physiological and pathological actions in the CNS including: fever; the induction of complement proteins, acute phase molecules and β-amyloid precursor protein (APP) synthesis; recruitment of leukocytes into the CNS; antiproliferative actions on a number of human tumors; and the regulation of glial cell growth and proliferation (11). Despite these findings, the normal physiological actions of IL-1 in the CNS remain unclear. IL-1 expression is highest in hypothalamus and hippocampus, with lower amounts in the brain stem and cortex (11). Activated microglia are the primary source in the CNS, although both neurons and astrocytes appear to synthesize IL-1. CNS concentrations of IL-1 are increased following head injury, CNS infections, or diseases associated with damage to the BBB (11). Infusion of IL-1 into rat brain results in glial activation and neuronal loss (23). *In vitro* studies demonstrate both neurotrophic and toxic actions of IL-1; low concentrations of IL-1 are neurotrophic and protect against excitotoxicity, whereas high concentrations are detrimental to long-term survival of hippocampal neurons (24). The recent development of mice deficient in interleukin-converting

enzyme (ICE; interleukin-1β-convertase), which are overtly normal but do not produce IL-1β following exposure to lipopolysaccharide (LPS), should facilitate understanding of IL-1 under both physiological and pathological conditions (25).

Undesired production of IL-1 may be regulated through inhibition of its synthesis or release, or by IL-1 receptor blockade (26). IL-1β is synthesized as an inactive precursor and selectively cleaved intracellularly by ICE to the biologically active form. ICE is a member of a cysteine protease family that includes ced-3, the programmed cell death factor in *C. elegans* (27). Four human ICE mRNA isoforms were identified recently (28). Various classes of ICE inhibitors have been identified and reviewed recently (26, 29). Prototypical examples include both reversible (1, K_i = 76 nM, monocyte IC_{50} = 2.7 μM) and irreversible (2, monocyte IC_{50} = 3 μM) inhibitors. CrmA, a protein encoded in the cowpox virus serpin, is a potent inhibitor of ICE, and has been shown to protect chicken dorsal root ganglia against cell death (30).





Various agents inhibit IL-1 release by both known and unknown mechanisms. Glucocorticoids decrease IL-1 mRNA stability, ultimately leading to a decrease in its release (31). Other classes of agents also inhibit IL release and have been reviewed recently (26, 32).

Endogenous IL-1ra exhibits no biological action of its own, but blocks the actions of IL-1α and β. Central injections of IL-1ra significantly reduced the extent of neuronal damage caused by either middle cerebral artery occlusion or NMDA receptor activation (33), two well-defined models of acute neurodegeneration. The degree of protection provided by IL-1ra was comparable to that obtained with MK-801, an NMDA receptor antagonist that is considered a benchmark neuroprotective agent in CNS ischemic injury models. Protection was not observed following treatment with the monoclonal antibody to IL-1R type II (10). Recombinant forms of soluble IL-1 type I receptor, which binds circulating IL-1, have been used in various animal models as well as in clinical trials to block effects of IL-1 (32). Recombinant human IL-1ra (Antril™) is undergoing clinical trials for treatment of rheumatoid arthritis and chronic myelogenous leukemias (32). In phase III clinical trials, Antril tended to decrease mortality in sepsis patients, however the results were not statistically significant (34).

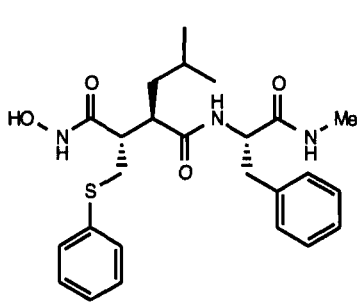
IL-6 is a multifunctional cytokine that induces the differentiation and proliferation of both normal and malignant cells, mediates the pathogenesis of shock and sepsis, induces acute phase proteins, and inhibits tumor growth (11). High concentrations of IL-6 have been reported in patients suffering from acute CNS infections, AD, MS, AIDS related dementia and lupus erythematosus. Activated microglia and astrocytes are the primary sources of IL-6 in the CNS. *In vitro* studies support a wide-range of biological actions of IL-6 including neurotrophic, neuroprotective, and toxic actions. IL-6 protects against neuronal damage following local infusion of NMDA into rat striatum (35) and ischemic damage (9). In contrast, transgenic mice overexpressing IL-6 demonstrate neuropathological manifestations that include neurodegeneration, astrocytosis, and induction of acute phase proteins (36).

TNFs represent the third family of cytokines associated with neurodegenerative diseases. TNF exists as either the α or β isoform, and both isoforms are synthesized as precursors that require specific processing to yield active peptide. TNF-α precursor is anchored onto plasma

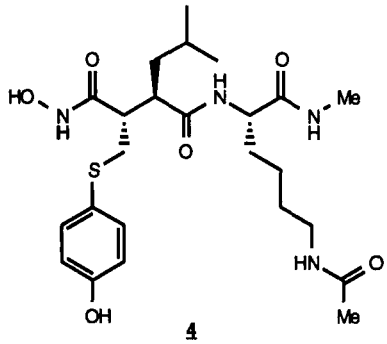
membrane from where it is cleaved by an unidentified protease (see below)(11). The precursor of TNF-β is cleaved within the rough endoplasmic reticulum and the mature form is released by secretory granules upon fusion with cell membrane. Two distinct TNF receptors, p55 (TNF-R1) and p57 (TNF-R2), have been identified (37). Aside from red blood cells, all cells express TNF receptors. Using anti-TNF receptor antibodies, studies demonstrated that the p55 receptor is responsible for the majority of pleiotropic actions of TNF, including cytotoxicity (38, 39).

Microglial cells and astrocytes synthesize both isoforms of TNF. *In vitro*, both isoforms are toxic to glial cells and oligodendrocyte cultures (40). TNF-α induced myelin degradation in organotypic cultures derived from CNS tissue (41). TNF-α and β potently induce MHC class I and II antigen expression, IL-1 and IL-6 release, fever, enhanced secretion of complement proteins, and elimination of virus-infected cells. Elevated levels of TNF-α and β have been reported in various CNS infections, including HIV-1 and meningitis, and following brain injury.

TNF concentrations may be regulated through inhibition of its processing or release mechanisms. TNF-α processing can be inhibited in LPS-treated monocytes by a series of hydroxamic acid-based inhibitors (**3**, IC_{50} = 180 nM; **4**, IC_{50} = 230 nM) (42, 43). In addition, **3** inhibited TNF-α production in LPS-treated mice (IC_{50} = 3-10 mg/kg, sc) (42). Although a specific enzyme(s) responsible for TNF-α processing has not been identified, the sequence spanning the scissile site in the TNF-α precursor and the effectiveness of selected hydroxamate-based inhibitors suggest that the TNF-α converting enzyme resembles a matrix metalloproteinase. Inhibitors of non-matrix metalloproteinases such as angiotensin converting enzyme or enkephalinase have no effect on inhibiting LPS-stimulated TNF-α production (43).

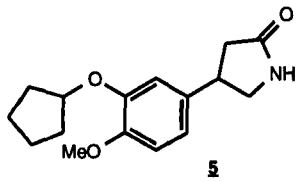


3



4

Increasing intracellular cAMP can suppress TNF release. Various classes of phosphodiesterase (PDE) inhibitors increase cAMP concentrations and thereby inhibit TNF production (44, 45). In general, selective inhibitors of PDE type IV (**5**, rolipram) are the more potent in suppressing TNF release than are inhibitors of PDE type III (44). Alternatively, overexpression of soluble forms of TNF receptors inhibit TNF-α-mediated effects both *in vitro* and *in vivo* (46).

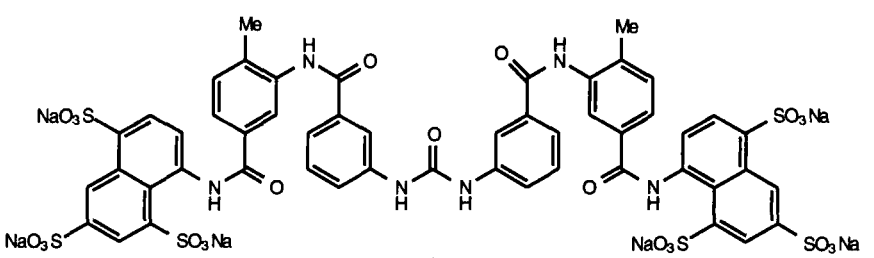


5

Complement - Activation of the complement system either through the alternative or classical pathway is an important defense mechanism against invading organisms and cells (47, 48). The alternative pathway provides a non-specific innate immunity that can be activated directly by an organism's cell membrane, and does not require involvement of antibodies. The classical pathway provides a more specific adaptive immunity, requiring interaction of an antigen with antibody for activation. Both pathways, which eventually converge, involve a highly regulated cascade of events in which discrete interactions of soluble proteins catalyze subsequent steps of the process. The end results of complement activation are formation of membrane attack complex (MAC) and recruitment of an inflammatory response, which together operate to lyse and remove the offending organism. MAC can also stimulate production of inflammatory mediators, inducing reactive oxygen radicals, arachidonic acid metabolites, and cytokines. Under normal conditions, the complement system is tightly controlled against self-attack through the presence of plasma or membrane-bound regulatory proteins. Overactivation of the complement system has been implicated as a pathological factor in various peripheral-based diseases and conditions (47) including rheumatoid arthritis, adult respiratory distress syndrome (ARDS), and bioincompatibility, as well as in various CNS disorders (49, 50).

In normal CNS tissue, complement proteins are detected at low levels using immunohistochemical analyses. However, these proteins are significantly upregulated in CNS tissues affected by various neurodegenerative disorders or by injury (51, 52). mRNAs for complement have been identified in brain tissue, and may be localized to activated microglia cells and possibly a subset of neurons (51). Recent studies have reported expression of various complement proteins from human glioma cell lines and from activated rat microglia cells (51). mRNA for certain complement proteins have been detected in primary astrocytes as well as human astrocytoma and glioma cell lines (53). Complement activation is not likely the cause of CNS diseases, but may contribute to the pathogenic processes. Inhibiting the complement activity may be of benefit in retarding the progressive neurodegeneration inherent in certain diseases.

As the complement cascade is composed of a number of tightly regulated enzymatic processes, pharmacological agents that inhibit at various points within the cascade have been identified. Compounds that inhibit either the classical or alternative pathways have been reviewed (54, 55). Structurally, these compounds range from polypeptides (MW > 30 KD) to various aromatic or heterocyclic molecules of low molecular weight (56, 57). Many of these compounds reduced complement-related pathology in animals models. However, a majority of these compounds are non-specific weak inhibitors, and possess toxicity at doses required for significant complement inhibition. Several compounds such as suramin (**6**) and ε-aminocaproic acid, inhibitors of the classical pathway, have been tested in the clinic. Endogenous inhibitors of complement, e.g. the soluble form of the membrane inhibitor CR1, are available in recombinant form (58). These large proteins have demonstrated efficacy in ameliorating complement-mediated injury in animal models.



6

DISEASES ASSOCIATED WITH NEUROINFLAMMATION

Alzheimer's Disease - AD is a progressive neurodegenerative disorder of unknown etiology that is characterized pathologically by the presence of extracellular senile plaques and intracellular neurofibrillary tangles (59). Activated microglia are located within or near the dystrophic neurons that are associated with senile plaques. The major component of plaques is amyloid β-peptide (Aβ), a 39- to 43- amino acid peptide that is cleaved from APP. Mutations in the gene encoding APP have been associated with some forms of familial AD. A number of studies have demonstrated that synthetic Aβ peptide is directly toxic to cultured neurons, although the mechanisms underlying this neurotoxicity are not understood (60, 61).

The pathology of AD is not characteristic of inflammation, in that it lacks evidence of edema or neutrophil invasion. However, mounting evidence indicates an increase in markers of inflammatory activity in the brains of AD patients (62). Activated microglial cells that surround senile plaques express class II major histocompatibility antigen, HLA-DR (5). Numerous immunohistochemical studies have detected increased levels of acute phase reactants, complement, complement receptors, cytokines (particularly TNF, IL-1 and IL-6) and cytokine receptors that colocalize with the senile plaques (51). Acute phase reactants such as α-antichymotrypsin (α-ACT) are elevated in serum and cerebral spinal fluid (CSF) of AD patients, and in senile plaques. Recent *in vitro* studies indicate that α-ACT promotes Aβ fibril formation, a structural feature of Aβ that is believed to impart neurotoxicity (63). Furthermore, IL-1 induces expression of α-ACT from human astrocyte cultures (64). IL-6 can stimulate the synthesis of APP from cultured neuronal cells, which in turn can be processed to Aβ (65).

Complement proteins associated with the classical pathway have been identified in AD brains (66). Dystrophic neurons surrounding senile plaques stain for MAC, suggesting that complement-mediated cell lysis may be occurring in AD. MAC staining is also detected in neurofibrillary tangles and in neuropil threads (66). MAC is not found in regions surrounding diffuse plaques, considered to be the precursor to the more dense senile plaque. The presence of immunoglobulins (Ig), a potential initiator of complement activation, in AD brain is somewhat controversial, and a lack of correlation between the localization of Ig immunoreactivity and dystrophic neurons and plaques staining for MAC has been noted (66). Endogenous complement inhibitors such as CD59 are increased in AD, suggesting a response by the tissue to complement insult (67).

Aβ peptide binds to C1q, the first component of the classical complement cascade (68), and activates complement in an antibody-independent manner (66). The activation is suggested to occur upon binding of Aβ to the collagen-like tail domain of C1q, a site that is distinct from the globular head region that interacts with Ig (69). The separate sites of interaction suggest the possibility of selectivity inhibiting Aβ-induced activation without interfering with Ig-mediated complement activation, and thereby preventing compromise of the peripheral immune system.

Brain cells appear to be the source of complement proteins since diffusion of these plasma-based proteins through the BBB is not observed. In addition, message for various complement proteins is increased in AD brains (51). Microglial cells in culture synthesize complement proteins, and their production is enhanced by treatment with Aβ (70).

Clinical data are emerging to support the presence of an inflammatory response that may contribute to the neuropathology of AD. An inverse relationship between use of anti-inflammatory medication and AD incidence in patients with rheumatoid arthritis has been noted in several retrospective studies (71). Co-twin studies support the inverse relationship between the onset of AD and prior use of various anti-inflammatory medications such as corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs) (72). Additional retrospective studies suggest NSAIDs may provide a protective role against onset of AD (73). Indomethacin slowed

decline of cognitive function in mild to moderately affected AD patients over a six-month period relative to a control group (74). Furthermore, a study in leprosy patients treated with the anti-inflammatory agent, dapsone, had a lower incidence of AD relative to patients not taking the drug (75).

AIDS Dementia - AIDS dementia complex (ADC) encompasses a broad range of cognitive, motor, and behavioral dysfunctions frequently observed in persons infected with the HIV-1 virus. Although the gross pathology of the ADC brain is variable, subcortical lesions are prominent, accompanied by an accumulation of mononuclear cells, multinucleated giant cells, reactive astrogliosis, loss of neurons and the destruction of white matter (white matter pallor). Detailed clinical and pathological findings have been reviewed (76 - 78).

Several theories exist to explain how the virus enters and then affects the brain (76). Cells in the CNS that harbor HIV-1 appear to be blood-derived macrophages, microglia, and multinucleated giant cells. Since there is little evidence for direct infection of the CNS by cell-free virus, infiltration by HIV-infected cells is proposed to be the mechanism. Relatively small numbers of HIV-1-infected cells are sufficient to induce gross brain pathology. Mounting evidence implicates cytokines, especially IL-1, TNF-α, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor–β (TGF-β), in the inflammatory and immune responses of ADC. The HIV-1 envelope protein, gp120, stimulate IL-6 and TNF-α release (79). Elevated levels of IL-1, TNF-α, and IL-6 have been detected in the CSF of ADC patients. Enhanced expression of IL-1, TGF-β, and TNF-α proteins has been observed in ADC brains (80). IL-1 can stimulate the HIV-1 enhancer in human T-cells (81). TNF-α and IL-6, either separately or in combination, have been reported to activate and enhance HIV-1 replication in macrophages (82). Similarly, GM-CSF, a factor that can induce the activation of microglia in the CNS, has been demonstrated to enhance production of HIV-1 in primary monocytes (83). In contrast, an immunosuppressive cytokine, TGF-β, has been demonstrated to suppress HIV replication in primary macrophages (84).

At present, drugs that inhibit HIV-1 replication in the CNS appear to be beneficial in treating ADC. Detailed descriptions of their structures, therapeutic doses and side effects have been reviewed (85, 86). Nucleoside inhibitors of HIV-1 reverse transcriptase (RT) are relatively hydrophilic and exhibit poor CNS penetration, thereby requiring relatively high doses to achieve therapeutic benefits. Of the RT inhibitors, zidovudine (ZDV) exhibits the highest CSF:plasma ratio. ADC patients treated with high doses of ZDV demonstrated improved neuropsychological parameters (76).

Multiple Sclerosis - MS is an inflammatory, demyelinating disease whose pathological hallmark is the appearance of large demyelinated lesions found within the white matter, the optic nerves, cerebellum, brainstem, and spinal cord. Early stages of the disease are associated with a relapsing and remitting (R-R) course, which is frequently followed by a progressive stage during which brainstem involvement can induce permanent disabilities. Details of the pathology and symptomatology of MS have been reviewed (87 - 90).

At present, the cause of MS is unknown although both environmental and genetic factors are implicated. A disturbance in regulation of the immune system is proposed to underlie the initiation and progression of the pathology. Adhesion molecules, MHC II-expressing reactive microglia, cytokines, macrophages, and T lymphocytes have been identified as factors contributing to the pathological process. Although a responsible autoantigen has not been identified, myelin basic protein (MBP) and proteolipid protein are proposed candidates (91).

Broad spectrum immunosuppressants and INF-β derivatives are widely used in treating MS. Immunosuppressive agents such as glucocorticoids reduce the inflammatory process through a number of potential mechanisms including induction of lipocortin secretion, inhibition of TNF-α, IL-6, IFN-γ and IL-1α and 1β secretion, as well as decrease in IL-1α and IL-1β

mRNA stability (31). These agents have proven useful for short term improvements in neurological function, although toxic side effects preclude their prolonged use.

The mechanism underlying the beneficial effects of INF-β in MS is not completely understood, although it is proposed to augment T-cell suppresser function, inhibit INF-γ production and inhibit INF-γ-induced MHC class II expression (14). Recombinant human INF-β-1β (Betaseron) is effective in treating R-R MS (92). Betaseron differs from INF-β by possessing a serine at position 17 to improve stability and having no glycosylated sites. Low dose treatment with rhINF-β-1α, which possesses the identical sequence as INF-β, significantly reduced the number and area of lesions. To date, these are the only agents demonstrated clinically to both reduce the relapse rate and alter the course of the disease. Recent results indicate that rINF-β-1α reduced the progression of disability of R-R MS (93).

The development of the experimental allergic encephalomyelitis (EAE) animal model has greatly facilitated the study of agents that interfere with specific immunopathogenic mechanisms associated with MS. Comprehensive discussions of agents studied in this model have been published (88). Clinical trials are underway using monoclonal antibodies directed toward antigenic peptides (MBP), T-cell surface proteins (CD4) and the receptor itself, adhesion molecules, cytokines, and MHC class II antigens (88).

Conclusion - Significant advances have been made within the last decade to support the involvement of inflammatory mechanisms in the pathogenesis of various neurodegenerative diseases. Additional studies are required to understand whether immune function in the CNS is fundamentally different from that in the periphery, or whether the apparent differences may be attributed to the unique environment of the brain that modifies the immune response. The efficacy of indomethacin in a small clinical study to slow the progression of AD is compelling. Other anti-inflammatory drugs that have been used to treat systemic chronic inflammatory disease will be interesting candidates for clinical trials in neurodegenerative diseases. Beyond empirical testing of these drugs, additional studies are necessary to determine whether common inflammatory mechanisms are operating across neurodegenerative diseases. Identifying and developing more specific agents that block inflammatory pathways that are exclusive in the brain may have the benefits of improved efficacy and reduced toxicity.

References

1. L.A. Lampson, Trends Neurosci., 10, 211 (1987).
2. P.S. Aisen and K.L. Davis, Am. J. Psychiatry, 151, 1105 (1994).
3. L. Brachova, L.-F. Lue, J. Schultz, T.E. Rashidy and J. Rogers, Molec. Brain Res., 18, 329 (1993).
4. R.B. Banati, J. Gehrmann, P. Schubert and G.W. Kreutzberg, GLIA, 7, 111 (1993).
5. P.L. McGeer, T. Kawamata, D.G. Walker, H. Akiyama, I. Tooyama and E.G. McGeer, GLIA, 7, 84 (1993).
6. V.H. Perry, P.-B. Anderson and S. Gordon, Trends Neurosci., 16, 268 (1993).
7. Z. Fabry, C.S. Raine and M.N. Hart, Immunol. Today, 15, 218 (1994).
8. S.J. Hopkins and N.J. Rothwell, Trends Neurosci., 18, 83 (1995).
9. N.J. Rothwell and S.J. Hopkins, Trends Neurosci., 18, 130 (1995).
10. N.J. Rothwell and J.K. Relton, Neurosci. Biobehav. Rev., 17, 217 (1993).
11. B. Schobitz, E.R.D. Kloet and F. Holsboer, Prog. Neurobiol., 44, 397 (1994).
12. C.A. Dinarello, FASEB J., 8, 1314 (1994).
13. G. Uze, G. Lutfalla and K.E. Mogensen, Journal of Interferon and Cytokine Research, 15, 3 (1995).
14. B. Weinstock-Guttman, R.M. Ransohoff, P. Kinkel and R.A. Rudick, Ann. Neurol., 37, 7 (1995).
15. A. Ljungdahl, T. Olsson, P.H.V.d. Meide, R. Holmdahl, L. Kareskog and B. Hojeberg, J. Neurosci. Res., 24, 451 (1989).
16. C. Reyes-Vazquez, B. Prieto-Gomez, J.A. Georgiades and N. Dafney, Int. J. Neurosci., 25, 113 (1984).
17. K.R. Huff and L.L.V. Ibric, Soc. Neurosci. Abst., 15, 511 (1989).
18. S.S. Raissdana, M.E. Barish, N.B. Mandorf and J. Kerline, Soc. Neurosci. Abs., 15, 18 (1989).
19. S. Baron, D. Weigent, G.J. Stanton and J. Peterson, Antivir. Res., 1, 173 (1985).

20. W.P. Arend, J. Clin. Invest., 88, 1445 (1988).
21. K.A. Deyerle, J.E. Sims, S.K. Dower and M.A. Bothwell, J. Immunol, 149, 1657 (1992).
22. G. Luheshi, Am. J. Physiol., 265, E585 (1993).
23. D. Giulian, GLIA, 7, 102 (1993).
24. D.M. Araujo and C.W. Cotman, Third IBRO World Conference on Neuroscience, 243, (1991).
25. P. Li, H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, E. Towne, D. Tracey, S. Wardwell, F.-Y. Wei, W. Wong, R. Kamen and T. Seshadri, Cell, 80, 401 (1995).
26. D. Tracey, Ann. Rep. Med. Chem., 29, 205 (1994).
27. J. Yuan, S. Shaham, S. Ledoux, H.M. Ellis and H.R. Horvitz, Cell, 75, 641 (1993).
28. E.S. Alnemri, T. Fernandes-Alnemri and G. Litwack, J. Biol. Chem., 270, 4312 (1995).
29. N.A. Thornberry and S.M. Molineaux, Protein Sci., 4, 3 (1995).
30. V. Gagliardini, P.A. Fernandez, R.K.K. Lee, H.C.A. Drexler, R.J. Rotello, M.C. Fishman and J. Yuan, Science, 263, 826 (1994).
31. Y. Amano, S.W. Lee and A.C. Allison, Mol. Pharmacol., 43, 176 (1992).
32. C.A. Dinarello and R.C. Thompson, Immunol. Today, 12, 404 (1991).
33. J.K. Relton and N.J. Rothwell, Brain Res. Bull., 585, 135 (1992).
34. C.J. Fisher, J.F. Dhainaut, S.M. Opal, J.P. Pribble, R.A. Balk, G.J. Slotman, T.J. Iberti, E.L. Rackow, M.J. Greenman, H.D. Reines, M.P. Shelly, B.W. Thompson, J.F. LaBrecque, M.A. Catalano, W.A. Knaus and J.C. Sadoff, J. Am. Med. Assoc., 271, 1836 (1994).
35. S. Toulmond, X. Vige, D. Fage and J. Benavides, Neurosci. Lett., 144, 49 (1992).
36. I.L. Campbell, C.R. Abraham, E. Masliah, P. Kemper, J.D. Inglis, M.B.A. Oldstone and L. Mucke, Proc. Natl. Acad. Sci. (USA), 90, 10061 (1993).
37. H.P. Hohmann, M. Brockhaus, P.A. Baeuerle, R. Remy, R. Kolbeck and A.P.G.M.v. Loon, J. Biol. Chem, 265, 22409 (1990).
38. L.A. Tartaglia, R.F. Weber, I.S. Figari, C. Reynolds, M.A. Palladino and D.V. Goedeel, Proc. Natl. Acad. Sci (USA), 88, 9292 (1991).
39. L.A. Tartaglia, T.M. Ayres, G.H.W. Wong and D.V. Goeddel, Cell, 74, 945 (1993).
40. K. Selmaj, C.S. Raine, M. Farooq, W.T. Norton and C.F. Brosnan, J. Immunol., 157, 1522 (1991).
41. K. Selmaj and C.S. Raine, Ann. Neurol., 23, 333 (1988).
42. G.M. McGeehan, J.D. Becherer, R.C. Bast, C.M. Boyer, B. Champion, K.M. Connolly, J.G. Conway, P. Furdon, S. Karp, S. Kidao, A.B. McElroy, J. Nichols, K.M. Pryzwansky, F. Schoenen, L. Sekut, A. Truesdale, M. Verghese, J. Warner and J.P. Ways, Nature, 370, 558 (1994).
43. A.J.H. Gearing, P. Beckett, M. Christodouiou, M. Churchill, J. Clements, A.H. Davidson, A.H. Drummong, W.A. Galloway, R. Gilbert, J.L. Gordon, T.M. Leber, M. Mangan, K. Miller, P. Nayee, K. Owens, S. Patel, W. Thomas, G. Wells, L.M. Wood and K. Woolley, Nature, 370, 555 (1994).
44. F.U. Schade and C. Schudt, Eur. J. Pharmacol., 230, 9 (1993).
45. R.M. Strieter, D.G. Remick, P.A. Wari, R.N. Spengler, I. J.P. Lynch, J. Larrik and S.L. Kunkel, Biochem. Biophys. Res. Commun., 155, 1230 (1988).
46. K. Selmaj, W. Papierz, A. Glabinski and T. Kohno, J. Neuroimmol., 141, 135 (1995).
47. B.P. Morgan, Eur. J. Clin. Invest., 24, 219 (1994).
48. M.M. Frank and L.F. Fries, Immunol. Today, 12, 322 (1991).
49. P.L. McGeer, H. Akiyama, S. Itagaki and E.G. McGeer, Can. J. Neurol. Sci., 16, 516 (1989).
50. P.L. McGeer, E.G. McGeer, T. Kawamata, T. Yamada and H. Akiyama, Can. J. Neurol. Sci., 18, 376 (1991).
51. S.A. Johnson, M. Lampert-Etchells, G.M. Pasinetti, I. Rozovsky and C.E. Finch, Neurobiol. Aging, 13, 641 (1992).
52. G.M. Pasinetti, S.A. Johnson, I. Rozovsky, M. Lampert-Etchells, D.G. Morgan, M.N. Gordon, T.E. Morgan, D. Willoughby and C.E. Finch, Exp. Neurol., 118, 117 (1992).
53. D.G. Walker and P.L. McGeer, Neurosci. Lett., 157, 99 (1993).
54. S.S. Asghar, Pharmacol. Rev., 36, 223 (1984).
55. W.K. Hagmann and R.D. Sindelar, Ann. Rep. Med. Chem., 27, 199 (1992).
56. J.M. Weiler, R.E. Edens, R.J. Linhardt and D.P. Kapelanski, J. Immunol., 148, 3210 (1992).
57. S. Miyagawa, R. Shirakura, G. Matsumiya, S. Kitagawa, N. Fukushima, S. Nakata, S. Nakano, H. Kitamura, M. Matsumoto, T. Seya and H. Matsuda, Transplant. Proc., 24, 483 (1992).
58. H.F. Weisman, T. Bartow, M.K. Leppo, J. H.C. Marsh, G.R. Carson, M.F. Concino, M.P. Boyle, K.H. Roux, M.L. Weisfeldt and D.T. Fearon, Science, 249, 146 (1990).
59. D.J. Selkoe, Neuron, 6, 487 (1991).
60. A.R. Korotzer, C.J. Pike and C.W. Cotman, Brain Res., 624, 121 (1993).
61. L. Meda, M.A. Cassatella, G.I. Szendrei, L. Otvos, P. Baron, M. Villalba, D. Ferrari and F. Rossi, Nature, 374, 647 (1995).
62. P. Eikelenboom, S.-S. Zhan, W.A.v. Gool and D. Allsop, Trends Pharmacol. Sci., 15, 447 (1994).
63. J. Ma, A. Yee, H.B. Brewer, S. Das and H. Potter, Nature, 372, 92 (1994).
64. S. Das and H. Potter, Neuron, 14, 447 (1995).

65. P. Vandenabeele and W. Fiers, Immunol. Today, 12, 217 (1991).
66. P.L. McGeer, J. Rogers and E.G. McGeer, Alzheimer Disease and Associated Disorders, 8, 1 (1994).
67. P.L. McGeer, D.G. Walker, H. Akiyama, T. Kawamata, A.L. Guan, C.J. Parker, N. Okada and E.G. McGeer, Brain Res., 544, 315 (1991).
68. R.B. Sim and K.B.M. Reid, Immunol. Today, 12, 307 (1991).
69. H. Jiang, D. Burdick, C.G. Glabe, C.W. Cotman and A.J. Tenner, J. Immunol., 152, 5050 (1994).
70. S. Haga, K. Ikeda, M. Sato and T. Ishii, Brain Res., 601, 88 (1993).
71. P.L. McGeer and J. Rogers, Neurology, 42, 447 (1992).
72. J.C.S. Breitner, B.A. Gau, K.A. Welsh, B.L. Plassman, W.M. McDonald, M.J. Helms and J.C. Anthony, Neurology, 44, 227 (1994).
73. J.B. Rich, D.X. Rasmusson, M.F. Folstein, K.A. Carson, C. Kawas and J. Brandt, Neurology, 45, 51 (1995).
74. J. Rogers, L.C. Kirby, S.R. Hempelman, D.L. Berry, P.L. McGeer, A.W. Kaszniak, J. Zalinski, M. Cofield, L. Mansukhani, P. Willson and F. Kogan, Neurology, 43, 1609 (1993).
75. J. Schnabel, Science, 260, 1719 (1993).
76. P. Portegies, J. Acq. Immun. Defic. Syndrome, 7(Suppl. 2), S38 (1994).
77. J.E. Merrill and I.S.Y. Chen, FASEB J., 6, 2391 (1991).
78. R. Geleziunas, H.M. Schipper and M.A. Wainberg, AIDS, 6, 1411 (1992).
79. Z. Rosenberg and A.S. Fauci, FASEB J., 5, 2382 (1991).
80. E.N. Benvensite, *Cytokine Circuits in Brain. Implications for AIDS Dementia Complex*, in *HIV, AIDS and the Brain*, R.W. Price and S.W. Perry, Editor. 1994, Raven Press, Ltd.: New York. p. 71.
81. L. Osborn, S. Kunkel and G.J. Nabel, Proc. Natl. Acad. Sci. (USA), 86, 2336 (1989).
82. G. Poli, P. Bressler and A. Kinter, J. Exp. Med., 172, 151 (1990).
83. Y. Koyanagi, W.A. O'Brien, J.Q. Zhao, D.W. Golde, J.C. Gasson and I.S.Y. Chen, Science, 241, 1673 (1988).
84. G. Poli, A.L. Kinter, J.S. Justement, P. Bressler, J.H. Kehrl and A.S. Fauci, J. Exp. Med., 173, 589 (1991).
85. D.L. Romero, Ann. Rep. Med. Chem., 29, 123 (1994).
86. M.J. Kozal and T.C. Merigan, Curr. Opin. Infect. Dis., 7, 72 (1994).
87. B.G.W. Arnason and A.T. Reder, Clin. Neuropharmacol., 17, 495 (1994).
88. C.D. Dijkstra, C.H. Polman and F. Berkenbosch, Trends Pharmacol. Sci., 14, 124 (1993).
89. L. Jacobs and K.P. Johnson, Arch. Neurol., 51, 1245 (1994).
90. L. Steinman and P. Conlon, Bio/Technology, 13, 118 (1995).
91. C.S. Raine, Ann. Neurol., 36, S61 (1994).
92. I.M.S.S. Group, Neurology, 43, 655 (1993).
93. W.I. McDonald, Brit. Med. J., 310, 345 (1995).

Chapter 5. Nicotinic Acetylcholine Receptors: Molecular Biology, Chemistry and Pharmacology

Ian A. McDonald, Nicholas Cosford, Jean-Michel Vernier
Salk Institute Biotechnology/Industrial Associates, La Jolla, CA 92037

Introduction - In contrast to the immense effort directed towards the design and synthesis of selective muscarinic acetylcholine receptor (mAChR) agonists, nicotinic acetylcholine receptors (nAChR) have attracted very little attention. The pharmacology of nAChRs has been intensively studied, however, and is well summarized in a recent review (1). The current interest in the medicinal chemistry aspects of nAChRs is due, in part, to perceived beneficial effects of nicotine in CNS disorders such as Alzheimer's and Parkinson's diseases (2) and to notable advances in the molecular biology of these receptor complexes. This chapter will review nAChR medicinal chemistry efforts to date and will discuss the future of this field in which the potential to design safe, effective new therapies is closely linked to a growing knowledge of the existence of discrete receptor subtypes and their relationship to physiological disorders.

RECEPTOR STRUCTURE

Molecular Biology - nAChRs are pentameric, ligand-gated ion channels belonging to a family of receptor complexes including the glycine (3), 5-HT_3 (4) and $GABA_A$ (5) receptors. The best characterized are the Torpedo electric organ nAChR and the homologous vertebrate skeletal muscle receptor (6) which are composed of four individual subunits (α1, β, γ, δ or ε). In neuronal tissue, several genes have been identified that are homologous to muscle nAChR genes. Those coding for proteins with two adjacent cysteine residues at positions 192 and 193 are called α subunits and those that lack them are termed β subunits. Genomic libraries have yielded full length clones for α2-α9 and β2-β4 subunits from numerous animal species (7,8). Functional homo-oligomeric receptors (electrophysiology and/or pharmacology) have been observed with α7, α8 and α9 subunits alone (9-12). Hetero-oligomeric receptor complexes assemble when α2, α3 or α4 subunits are separately coexpressed with either β2 or β4 subunits to form a pentameric structure (13-17). The α5 subunit, in combination with α4 or β2 in oocytes gives no conductance signal in voltage clamp and patch clamp electrophysiological experiments. However, when all three subunits are co-expressed, a new nAChR was observed with properties distinctly different from α4β2 (18). Neither the relevence of these recombinant nAChRs to, nor indeed the molecular composition of native human CNS receptors *per se*, is known with any certainty. Conceivably, endogenous α receptors may exist as homo-oligomeric complexes or might occur in any hetero-oligomeric combination of subunits in combination with other α and β combinations. For example, immunodepletion experiments suggest that chick ciliary ganglia contain a receptor complex which is composed of at least three different subunits - α3, α5 and β4 subunits (19). When chicken neuronal nAChRs are expressed in oocytes, two α4 subunits assemble with three β2 subunits to form a functional receptor complex (20). The

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

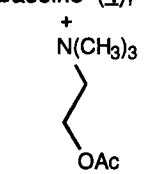
development of transgenic animal models may help elucidate the physiological roles of specific nAChR subunits. In a recent paper it was reported that transgenic mice lacking the β2 subunit exhibited abnormal avoidance learning behaviour (21).

nAChR Structural Studies - nAChRs have been the subject of an enormous number of structural studies over the past two decades. Excellent recent reviews (22-26) illustrate the prominence of the *Torpedo* ray nAChR in these studies. All nAChR subunits are membrane bound proteins and appear to have four membrane spanning regions, with both amino and carboxyl terminii on the extracellular side. Rapid freezing techniques, coupled with electron microscopy of acetylcholine-treated *Torpedo* ray membranes, elegantly visualized the gross nAChR pentameric structure (27). Affinity labeling studies have identified a number of amino acid residues in the ligand binding site of the *Torpedo* receptor complex (23, 28). Like other ligand-gated ion channels, nAChRs are believed to be replete with allosteric binding sites (29). An NMR study of the solution structure of α-bungarotoxin complexed to a small peptide portion of the *Torpedo* nACHR has been reported (30). Theoretical models of the nAChR binding site have been proposed (31).

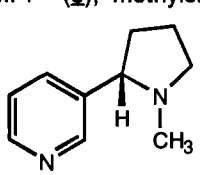
RECEPTOR AGONISTS

Therapeutic Opportunities - Selective nAChR agonists have great potential as therapeutic agents for a diverse group of central and peripheral nervous system disorders. Based largely on clinical observations with nicotine (**2**), coupled with a growing understanding of the pharmacology of nAChR agonists, these agents portend therapies for cognitive and attention disorders (32-34), Alzheimer's disease (2, 34-39), Parkinson's disease (2, 38, 40, 41), anxiety (42, 43), depression (44), smoking cessation (45), neuroprotection (46-49), schizophrenia (50, 51), analgesia (52, 53), Tourette's syndrome (1) and ulcerative colitis (54).

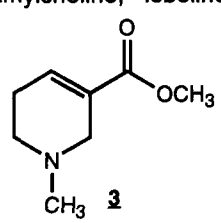
Classic nAChR Agonists and Antagonists - Early pharmacological characterization of nAChRs relied upon agonists, such as acetylcholine (**1**), (*S*)-nicotine (**2**), arecoline (**3**), anabaseine (**4**), DMPP (**5**), methylcarbamylcholine, lobeline (**6**) and cytisine (**7**); or



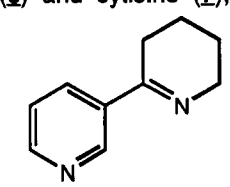
1



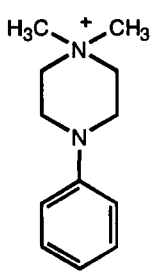
2



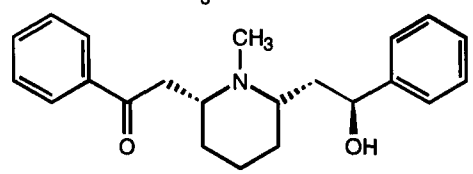
3



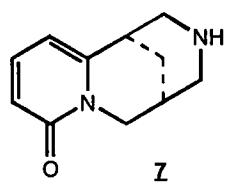
4



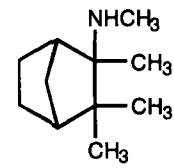
5



6

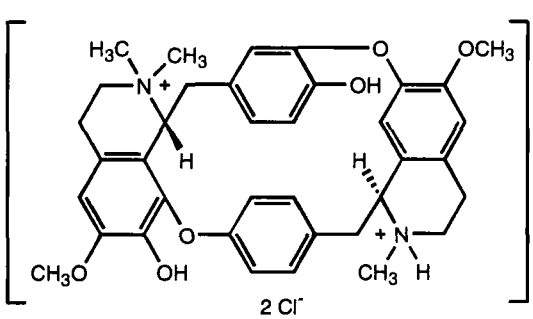


7

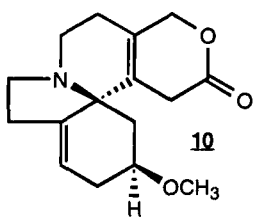


8

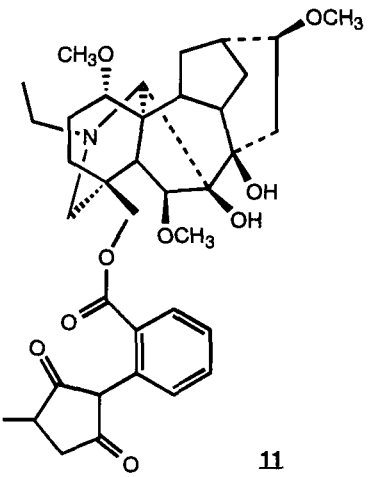
antagonists, such as hexamethonium, decamethonium, mecamylamine (**8**), *d*-tubocurarine (**9**), dihydro-β-erythroidine (**10**), methyllycaconitine (**11**), α-bungarotoxin and snake α-toxins, among others (1). A series of recent papers discussed a rapid isolation procedure for **11** and reported on the synthesis of several structural analogues (55-57). Although these ligands have been extremely valuable pharmacological tools they appear to have limited therapeutic potential which is probably due to unacceptable side-effects. For example, activation of ganglionic or muscle receptors by nicotine (**2**) is seriously dose-limiting and severely limits this drug as a candidate for the treatment of CNS disorders (58). One drug (metocurine, a methoxy derivative of **9**), however, is used clinically as a muscle relaxant (59), and a second (**6**) is in Phase II clinical trials for smoking cessation (60). Furthermore, **7** is continuing to be evaluated and has shown good activity in pharmacological models of



9



10

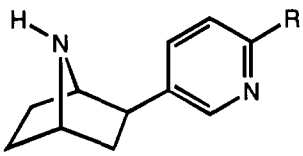


11

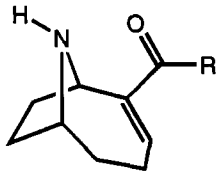
learning and memory (61) and analgesia (62), suggesting that nAChRs may play a role in the modulation of these processes. The growing understanding of the multiple nAChR complexes clearly points to the need for subtype selective ligands. In an important paper it was reported that **1**, **2**, **5** and **7** exert differential agonist effects on the recombinant receptor complexes α2β2, α3β2, α4β2, α2β4, α3β4 and α4β4, indicating that the discovery of such selective compounds is conceptually attainable (63).

nAChR Agonists, Recent Advances - Since the report (64) in 1992 that epibatidine (**12**), a 7-azanorbornane analogue of nicotine, possessed extraordinary analgesic properties and the demonstration in early 1994 that **12** was an exquisitely potent nAChR agonist (53, 65), many groups turned to the synthesis of this interesting molecule. Syntheses of racemic **12** (66-73), enantiomerically pure forms (74-76) and the derivatives **13** to **15** appeared (76). Interestingly, removal of the chlorine atom in **12** (i.e. **13**) had no effect on binding potency. The absolute configuration of **12** was determined to be 1*R*, 2*R*, 4*S* (75). Both enantiomers of **12** possess potent analgesic properties (77) and bind to [^{3}H]-**2** sites in rat brain with the same affinity (K_i = 55 pM). Since the analgesic effect is blocked by **8**, nAChRs are strongly implicated in the mechanism of action. Epibatidine (**12**), however, is a potent toxin (isolated

from the skin of a poisonous frog) and has an extremely narrow therapeutic index. The challenge is to separate the toxicity from its analgesic effect; whether both effects are mediated through nAChRs remains to be established.

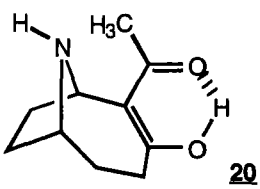


12 R=Cl
13 R=H
14 R=CH_3
15 R=I

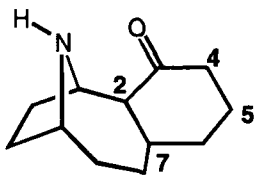


16 R=CH_3
17 R=CH_2CH_3
18 R=$CH_2CH_2CH_3$
19 R=$CH(CH_3)_2$

Another well known toxin, (+)-anatoxin-a (**16**), produced by the fresh water cyanobacterium *Anabaena flos aqua*, has attracted considerable chemical and biological interest. In four functional assays (78), **16** was found to be more potent than either **1** or **2**. Compound **16** potently stimulated stably expressed, recombinant α4β2 and α7 nAChRs, stimulated the release of (**1**) from hippocampal synaptosomes, and activated α-bungarotoxin-sensitive nAChRs using patch clamp techniques. Analogues **17** to **19**, in which the side chain of **16** has been modified, have been prepared and studied (79). Working with a series of 21 anatoxin analogues, a QSAR study led to clustering models with reasonable predictive properties (80). Conformationally biased (**20**) or constrained (**21** to **23**) analogues were reported (81, 82). Compound **20** was significantly less active than **16**; no biological data were furnished for **21**-**23**.

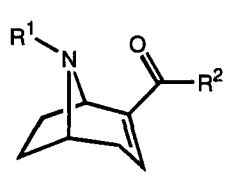


20

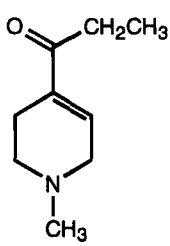


21 2,7-unsaturation
22 4,5-unsaturation, *cis*-ring fusion
23 4,5-unsaturation, *trans*-ring fusion

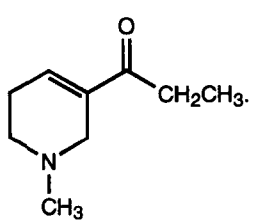
A synthesis of ferruginine (**24**) was reported (83) and this product and analogues (e.g. **25**) were claimed as nAChR agonists for the treatment of neurodegenerative diseases (84, 85). The binding affinities of a series of isoarecolones and arecolones for nAChRs and



24 R^1=CH_3, R^2=CH_3
25 R^1=H, R^2=OH



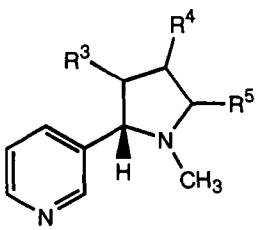
26



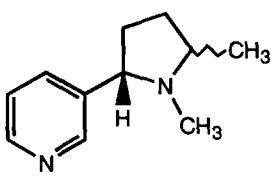
27

mAChRs were reported (86). The most selective compounds (**26** and **27**) in each series had K_i (nAChR) values of 26 nM and 6 nM, respectively, with selectivity relative to mAChR binding of >195 and 336, respectively.

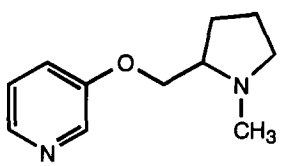
Although nicotine (**2**) has been known to chemists for decades, surprisingly little systematic SAR study has been undertaken with this small molecule. The synthesis and binding properties of a series of pyrrolidine-substituted nicotine analogues (**28**) was reported (87). An important observation was that the *trans* 5-β-CH_3 derivative (**29**, K_i = 35 nM) was significantly more potent than the *cis* 5-α-epimer (**30**, K_i = 1.2 μM). The same laboratory claimed in a patent application (88) a vast number of structures related to **31** which has potent affinity for the nAChR (K_i = 0.2 nM; [^{3}H]-**7**). The *N*-linked pyrrolidine (**32**; K_i = 49 nM) has also appeared in the patent literature (88). A high affinity, irreversible nAChR ligand (**33**, K_i = 70 nM) was described (90).



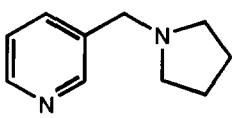
28



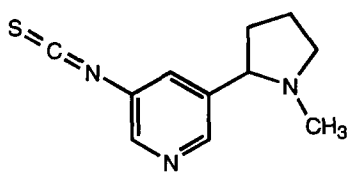
29 β-Isomer
30 α-Isomer



31

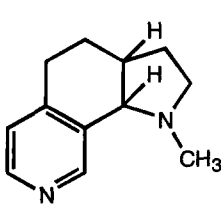


32

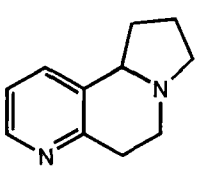


33

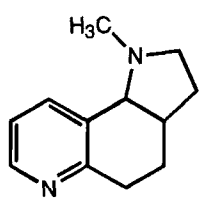
The conformationally restrained isoquinoline (+)-**34** was reported to possess antinociceptive properties *in vivo* but this compound had no appreciable affinity for nAChRs in binding experiments (91). In contrast, (-)-**34**, was a somewhat less potent antinociceptive agent, but bound to nAChRs with modest affinity. The (+)-isomer had the same relative stereochemistry as (*S*)-(-)-**2** (the natural form). Other tricyclic nicotine analogues (**35** to **37**) have been previously described; **35** and **36** had no appreciable biological activity (92, 93). The compound, **37**, however, which encompasses structural features of **2** and **16**, was very potent in binding (IC_{50} = 5 nM) and functional assays (94).



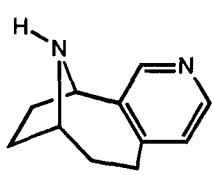
34



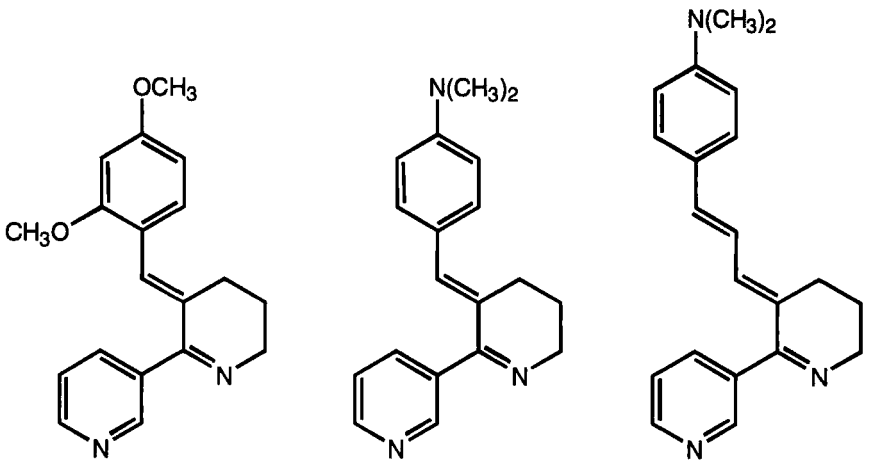
35



36



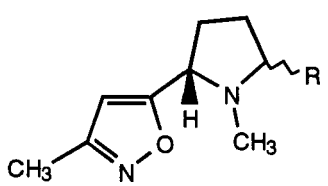
37



38 39 40

The anabaseine analogues **38** to **40** demonstrate the potential of designing selective nAChR agonists. These novel structural variants were found to have significant effects on nAChR receptor binding and functional properties. For example, whereas **4** was shown to be a strong partial agonist at α4β2 nAChRs expressed in oocytes, **38**-**40** were weak agonists. However, **38**-**40** were more potent agonists at α7 than **4**. The K_i values from binding studies with [^{3}H]-**7** (putatively indicating affinity for α4β2 nAChRs) and [^{125}I]-BTX (α7) were in accord with these observations (95). The pharmacology of these molecules is no less interesting. They were active in a model of passive avoidance in bilaterally nucleus basalis-lesioned rats, but were less potent than **2** or **4** (96). One compound (**38**) was shown to facilitate eyeblink classical conditioning which is believed to reflect activation of the septo-hippocampal cholinergic system (97). Finally, it was reported that **38** elicits cytoprotective activity in NGF-sensitive neuronal populations (98).

ABT 418 (**41**) further exemplifies the therapeutic potential of nAChR agonists which show selectivity for central receptor subtypes. This isoxazole derivative displaces [^{3}H]-**7** with a K_i = 4.2 nM (equipotent with **2**) and is postulated to show selectivity for α4β2 receptors (99). The (*S*)-enantiomer, like (*S*)-(**2**), is about 10-fold more potent than the (*R*)-isomer. Extensive SAR studies have been reported (99-101). Although **41** does not appear to have



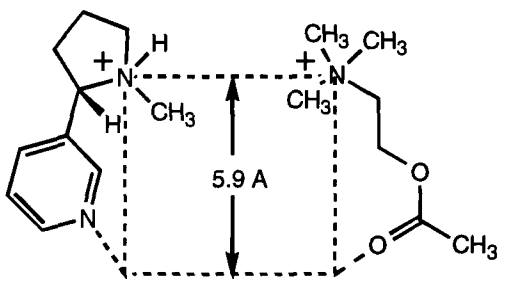
41 R=H
42 R=βCH3
43 R=αCH3

been studied in cells expressing functionally active, recombinant nAChR subunits, in PC12 cells patch clamp studies have demonstrated potent agonist activity (102). Extensive *in vivo* experiments clearly indicate that **41** has both cognition-enhancing and anxiolytic properties (102) which are convincingly mediated through a nAChR mechanism (blocked by **8**). Its

poor pharmacokinetic profile after oral administration, but excellent transdermal properties, led to the decision to develop this promising compound as a patch for the treatment of AD. In an apparent attempt to address the bioavailability issue (101), the 5'-methyl-substituted derivatives **42** and **43** (K_i = 71 nM and 5 μM respectively) were prepared; *in vitro* half lives of **42** and **43**, however, were not greatly improved.

Pharmacophore Modeling - Attempts to develop nAChR agonist/antagonist pharmacophores have been reported on several occasions. A conformational analysis approach led to the proposal that one of the key elements in the binding of ligands to the nAChR is a hydrogen bond between a receptor hydrogen donor group and an acceptor group in the ligand and is formed 5.9 A from the positively charged aliphatic nitrogen atom (103). In contrast, acetylcholine adopts a more compact conformation in the active site of the mAChR such that this distance is reduced to 4.4 A. A more elaborate nAChR model, which was developed by distance geometry methods, was in basic agreement with thess predictions (104). To date, however, these models out of necessity have considered the nAChR as a single complex. As subtype selective ligands are developed, more refined models will be forthcoming.

Beers and Reich pharmacophore (103)



2 **1**

Conclusion - The therapeutic potential of selective nAChR agonists today is reflective of the situation of 5-HT medicinal chemistry a decade ago. There are multiple receptor subtypes, it is clearly possible to design selective agonists (and antagonists), an old drug (**6**) is in Phase II clinical trials and the first of the newer drugs (**41**) has recently entered the clinic. Furthermore, there is a growing understanding of the physiological and pharmacological effects of drugs acting at nAChRs (105-108). The challenge today lies on several fronts. First, is the localization and characterization of endogenous receptor subtypes, and the need to demonstrate the link between receptor subtypes and particular diseases or physiological functions. Second, is the development of cell-based functional assays in which physiologically relevant, recombinant receptor complexes are used for drug screening and characterization. These multimeric receptor complexes present a significantly greater challenge to molecular and cellular biologists than the G-protein linked serotonin receptors. Finally, structure-activity relationship studies need to be developed for each receptor subtype and therapeutically useful compounds must be designed and synthesized. This short review has focused primarily on the design of ligands interacting at the acetylcholine binding site; clearly future drug development will consider both competitive and allosteric binding sites.

References

1. M. Williams, J.P. Sullivan and S.P. Arneric, Drug News & Perspectives, 7, 205 (1994).
2. E.K. Perry, C.M. Morris, J.A. Court, A. Cheng, A.F. Fairbairn, I.G. McKeith, D. Irving, A. Brown and R.H. Perry, Neuroscience, 64, 385 (1995).
3. H. Betz, Neuron, 5, 383 (1990).
4. A.V. Maricq, A.S. Peterson, A.J. Brake, R.M. Myers and D. Julius, Science, 254, 432 (1991).
5. W. Wisden and P.H. Seeburg, Curr. Opin. Neurobiol., 2, 263 (1992).
6. E.S. Deneris, J. Connolly, S.W. Rogers and R. Duvoisin, Trends Pharmacol. Sci., 12, 34 (1991).
7. P.B. Sargent, Annu. Rev. Neurosci., 16, 403 (1993).
8. A.B. Elgoyhen, D.S. Johnson, J. Boulter, D.E. Vetter and S. Heinemann, Cell, 79, 705 (1994).
9. Z.W. Zhang, S. Vijayaraghavan and D.K. Berg, Neuron, 12, 167 (1994).
10. S. Couturier, D. Bertrand, J.M. Matter, M.C. Hernandez, S. Betrand, N. Millar, S. Valera, T. Barkas and M. Ballivet, Neuron, 5, 847 (1990).
11. P. Seguela, J. Wadiche, K. Dineley-Miller, J.A. Dani and J.W. Patrick, J. Neurosci., 13, 596 (1993).
12. V. Gerzanich, R. Anand and J. Lindstrom, Mol. Pharmacol., 45, 212 (1994).
13. J. Boulter, J. Connolly, E. Deneris, D. Goldman, S. Heinemann and J. Patrick, Proc. Natl. Acad. Sci. U.S.A., 84, 7763 (1987).
14. M. Ballivet, P. Nef, S. Couturier, D. Rungger, C.R. Bader, D. Bertrand and E. Cooper, Neuron, 1, 847 (1988).
15. E.S. Deneris, J. Connolly, J. Boulter, E. Wada, K. Wada, L.W. Swanson, J. Patrick and S. Heinemann, Neuron, 1, 45 (1988).
16. K. Wada, M. Ballivet, J. Boulter, J. Connolly, E. Wada, E.S. Deneris, L.W. Swanson, S.F. Heinemann and J. Patrick, Science, 240, 330 (1988).
17. R.M. Duvoisin, E.S. Deneris, J. Patrick and S. Heinemann, Neuron, 3, 487 (1989).
18. J.A. Ramirez Latorre, X. Qu and L. Role, Soc. Neurosci. Abst., 19, 1533 (1993).
19. A.B. Vernallis, W.G. Conroy and D.K. Berg, Neuron, 10, 451 (1993).
20. R. Anand, W.G. Conroy, R. Schoepfer, P. Whiting and J. Lindstrom, J. Biol. Chem., 266, 11192 (1991).
21. M.R. Picciotto, M. Zoli, C. Lena, A. Bessis, Y. Lallemand, N. LeNovere, P. Vincent, E.M. Pich, P. Brulet and J.P. Changeux, Nature, 374, 65 (1995).
22. B.M. Conti-Tronconi, K.E. McLane, M.A. Raftery, S.A. Grando and M.P. Protti, Crit. Rev. Biochem. Mol. Biol., 29, 69 (1994).
23. A. Karlin, Curr. Opin. Neurobiol., 3, 299 (1993).
24. J.P. Changeux, Sci. Amer., 269, 58 (1993).
25. A. Devillers-Thiery, J.L. Galzi, J.L. Eisele, S. Bertrand, D. Bertrand and J.P. Changeux, J. Membrane Biol., 136, 97 (1993).
26. N. Unwin, Neuron, 10, (Suppl.), 31 (1993).
27. N. Unwin, Nature, 373, 37 (1995).
28. J.B. Cohen, S.D. Sharp and W.S. Liu, J. Biol. Chem., 266, 23354 (1991).
29. C. Lena and J.P. Changeux, Trends Neurosci., 16, 181 (1993).
30. V.J. Basus, G. Song and E. Hawrot, Biochem., 32, 12290 (1993).
31. V.B. Cockcroft, G.G. Lunt and D.J. Osguthorpe, Biochem. Soc. Symp., 57, 65 (1990).
32. C. Vidal, Drug. Devel. Res., 31, 120 (1994).
33. E.D. Levin, Psychopharmacology, 108, 417 (1992).
34. M.W. Decker and J.L. McGaugh, Synapse, 7, 151 (1991).
35. J.T. Greenamyre and W.F. Maragos, Cerebrovasc. Brain Metab. Rev., 5, 61 (1993).
36. S.P. Arneric and M. Williams in "Recent Advances in the Treatment of Neurodegenerative Disorders and Cognitive Dysfunction", Int. Acad. Biomed. Drug. Res., Karger, Basel, Vol 7, 1994 p. 58.
37. A. Nordberg, Neurochem. Int., 25, 93 (1994).
38. C.J. Smith and E. Giacobini, Rev. Neurosci., 3, 25 (1992).
39. C.M. van Duijn and A. Hofman, Br. Med. J., 302, 1491 (1991).

40. K.O. Fagerström, O. Pomerleau, B. Giordani, F. Stelson, Psychopharmacology, 116, 117 (1994).
41. J.A. Baron, Neurology, 36, 1490 (1986).
42. O.F. Pomerleau, Psychopharmacol. Bull., 22, 865 (1986).
43. J.D. Brioni, A.B. O'Neill, D.J.B. Kim and M.W. Decker, Eur. J. Pharmacol., 238, 1 (1993).
44. N. Breslau, M.M. Kilbey and P. Andreski, Arch. Gen. Psychiatr., 50, 31 (1993).
45. K.J. Palmer, M.M. Buckley and D. Faulds, Drugs, 44, 498 (1992).
46. C. Owman, K. Fuxe, A.M. Janson and J. Kahrstrom, Prog. Brain. Res., 79, 267 (1989).
47. P. Marin, M. Maus, S. Desagher, J. Glowinski and J. Prémont, NeuroReport, 5, 1977 (1994).
48. P.A. Lappchak, D.M. Araujo and F. Hefti, Neuroscience, 60, 293 (1994).
49. A. Akaike, Y. Tamura, T. Yokota, S. Shimohama and J. Kimura, Brain Res., 644, 181 (1994).
50. S. Mukherjee, S.P. Mahadik, A. Korenovsky, H. Laev, D.B. Schnur and R. Reddy, Schizophrenia Res., 12, 131 (1994).
51. P. Curzon, D.J. Kim and M.W. Decker, Pharmacol. Biochem. Behav., 49, 877 (1994).
52. C. Qian, T. Li, T.Y. Shen, L.L. Garahan, J. Eckman, T. Biftu and S. Ip, Eur. J. Pharmacol., 250, R13 (1993).
53. B. Badio and J.W. Daly, Mol. Pharmacol., 45, 563 (1994).
54. R.D. Pullan, J. Rhodes, S. Ganesh, V. Mani, J.S. Morris, G.T. Williams, R.G. Newcombe, M.A.H. Russell, C. Feyerabend, G.A.O. Thomas and U. Sawe, New Engl. J. Med., 330, 811 (1994).
55. P.A. Coates, I.S. Blagbrough, D.J. Hardick, M.G. Rowan, S. Wonnacott and B.V.L. Potter, Tetrahedron Lett., 35, 8701 (1994).
56. I.S. Blagbrough, P.A. Coates, D.J. Hardick, T. Lewis, M.G. Rowan, S. Wonnacott and B.V.L. Potter, Tetrahedron Lett., 35, 8705 (1994).
57. P.A. Coates, I.S. Blagbrough, M.G. Rowan, B.V.L. Potter, D.P.J. Pearson and T. Lewis, Tetrahedron Lett., 35, 8709 (1994).
58. N.L. Benowitz, Trends Cardiovasc. Med., 1, 315 (1992).
59. "Physicians' Desk Reference", 49th Ed., 1995, p. 940.
60. News item, BioWorld Today, Aug 17, 4 (1993).
61. M.W. Decker, M.J. Majchrzak and S.P. Arneric, Pharmacol. Biochem. Behav., 45, 571 (1993).
62. S.R. Hamann and W.R. Martin, Pharmacol. Biochem. Behav., 47, 197 (1994).
63. C.W. Luetje and J. Patrick, J. Neurosci., 11, 837 (1991).
64. T.F. Spande, H.M. Garraffo, M.W. Edwards, H.J.C. Yeh, L. Pannell and J.W. Daly, J. Am. Chem. Soc., 114, 3475 (1992).
65. J.P. Sullivan, M.W. Decker, J.D. Brioni, D. Donnelly-Roberts, D.J. Anderson, A.W. Bannon, C.H. Kang, P. Adams, M. Piattoni-Kaplan, M.J. Buckley, M. Gopalakrishnan and S.P. Arneric, J. Pharmacol. Exp. Ther., 271, 624 (1994).
66. D.F. Huang and T.Y. Shen, Tetrahedron Lett., 34, 4477 (1993).
67. C.A. Broka, Tetrahedron Lett., 34, 3251 (1993).
68. S.C. Clayton and A.C. Regan, Tetrahedron Lett., 34, 7493 (1993).
69. G. Pandey, T.D. Bagul and G. Lakshmaiah, Tetrahedron Lett., 35, 7439 (1994).
70. K. Sestanj, E. Melenski and I. Jirkovsky, Tetrahedron Lett., 35, 5417 (1994).
71. C. Szantay, Z. Kardos-Balogh, I. Moldvai, C. Szantay, E. Temesvari-Major and G. Blasko, Tetrahedron Lett., 35, 3171 (1994).
72. K. Senokuchi, H. Nakai, M. Kawamura, N. Katsube, S. Nonaka, H. Sawaragi and N. Hamanaka, Synlett, 343 (1994).
73. S.Y. Ko, J. Lerpiniere, I.D. Linney and R. Wrigglesworth, J. Chem. Soc. Chem. Commun., 1775 (1994).
74. S.R. Fletcher, R. Baker, M.S. Chambers, S.C. Hobbs and P.J. Mitchell, J. Chem. Soc. Chem. Commun., 1216 (1993).
75. S.R. Fletcher, R. Baker, M.S. Chambers, R.H. Herbert, S.C. Hobbs, S.R. Thomas, H.M. Verrier, A.P. Watt and R.G. Ball, J. Org. Chem., 59, 1771 (1994).
76. E.J. Corey, T.P. Loh, S. AchyuthaRao, D.C. Daley and S. Sarshar, J. Org. Chem., 58, 5600 (1993).

77. M.I. Damaj, K.R. Creasy, A.D. Grove, J.A. Rosecrans and B.R. Martin, Brain Res., 664, 34 (1994).
78. P. Thomas, M. Stevens, G. Wilkie, M. Amar, G.G. Lunt, P. Whiting, T. Gallagher, E. Pereira, M. Alkondon, E.X. Albuquerque and S. Wonnacott, J. Neurochem., 60, 2308 (1993).
79. P. Thomas, P.A. Brough, T. Gallagher and S. Wonnacott, Drug Dev. Res., 31, 147 (1994).
80. D.T. Manallack, D.D. Ellis, P.E. Thompson, T. Gallagher and D.J. Livingstone, Nat. Prod. Lett., 4, 121 (1994).
81. P.A. Brough, T. Gallagher, P. Thomas, S. Wonnacott, R. Baker, K.M.A. Malik and M.B. Hursthouse, J. Chem. Soc. Chem. Commun., 1087 (1992).
82. A. Hernandez and H. Rapoport, J. Org. Chem., 59, 1058 (1994).
83. H.M.L. Davies, E. Saikali and W.B. Young, J. Org. Chem., 56, 5696 (1991).
84. H.M.L. Davies and E. Saikali, US Patent 5,288,872 (1994).
85. W.S. Caldwell, H.M.L. Davies and P.M. Lippiello, US Patent 5,227,385 (1993).
86. J.S. Ward, L. Merritt, F.P. Bymaster and D.O. Calligaro, Bioorg. Med. Chem. Lett., 4, 573 (1994).
87. N.H. Lin, G.M. Carrera and D.J. Anderson, J. Med. Chem., 37, 3542 (1994).
88. M.A. Abreo, D.E. Gunn, N. Lin, R.L. Elliott, D.S. Garvey, S. Lebold and J.T. Wasicak, International Publication Number: WO 94/08992 (1994)
89. W.S. Caldwel and P.M. Lippiello, U.S. Patent 5,248,690 (1993).
90. K.D. Kim, N. Lerner-Marmarosh, M.Saraswati, A.S. Kende and L.G. Abood, Biochem. Pharmacol., 47, 1965 (1994).
91. W. Glassco, J. Suchocki, C. George, B.R. Martin and E.L. May, J. Med. Chem., 36, 3381 (1993).
92. T.E. Catka and E. Leete, J. Org. Chem., 43, 2125 (1978).
93. C.G. Chavdarian, J.I. Seeman and J.B. Wooten, J. Org. Chem., 48, 492 (1983).
94. D.B. Kanne and L.G. Abood, J. Med. Chem., 31, 506 (1988).
95. C.M. de Fiebre, E.M. Meyer, J.C. Henry, S.I. Muraskin, W.R. Kem and R.L. Papke, Mol. Pharmacol., 47, 164 (1995).
96. E.M. Meyer, C.M. de Fiebre, B.E. Hunter, C.E. Simpkins, N. Frauworth amd N.E.C. de Fiebre, Drug Dev. Res., 31, 127 (1994).
97. D.S. Woodruff-Pak, Y.T. Li and W.R. Kem, Brain Res., 645, 309 (1994).
98. E.J. Martin, K.S. Panickar, M.A. King, M. Deyrup, B.E. Hunter, G. Wang and E.M. Meyer, Drug Dev. Res., 31, 135 (1994).
99. D.S. Garvey, J.T. Wasicak, M.W. Decker, J.D. Brioni, M.J. Buckley, J.P. Sullivan, G.M. Carrera, M.W. Holladay, S.P. Arneric and M. Williams, J. Med. Chem., 37, 1055 (1994).
100. D.S. Garvey, J.T. Wasicak, R.L. Elliott, S.A. Lebold, A.M. Hettinger, G.M. Carrera, N.H. Lin, Y. He, M.W. Holladay, D.J. Anderson, E.D. Cadman, J.L. Raszkiewicz, J.P. Sullivan and S.P. Arneric, J. Med. Chem., 37, 4455 (1994).
101. N.H. Lin, Y. He, D.J. Anderson, J.T. Wasicak, R. Kasson, D. Sweeny and J.P. Sullivan, Bioorg. Med. Chem. Lett., 4, 2389 (1994).
102. S.P. Arneric, J.P. Sullivan, C.A. Briggs, D. Donnelly-Roberts, D.J. Anderson, J.L. Raszkiewicz, M.L. Hughes, E.D. Cadman, P. Adams, D.S. Garvey, J.T. Wasicak and M. Williams, J. Pharmacol. Exp. Ther., 270, 310 (1994).
103. W.H. Beers and E. Reich, Nature, 228, 917 (1970).
104. R.P. Sheridan, R. Nilakantan, J.S. Dixon and R. Venkataraghavan, J. Med. Chem., 29, 899 (1986).
105. M.W. Decker, J.D. Brioni, A.W. Bannon and S.P. Arneric, Life Sci., 56, 545 (1995).
106. B.M. Conti-Tronconi, K.E. McLane, M.A. Raftery, S.A. Grando and M.P. Protti, Crit. Rev. Biochem. Mol. Biol., 29, 69 (1994).
107. A. Nordberg, Neurochem. Int., 25, 93 (1994).
108. A.H. Glassman and L.S. Covey in "Critical Issues in the Treatment of Affective Disorders", Int. Acad. Biomed. Drug. Res., Karger, Basel, Vol 9, 1994 p. 21.

Chapter 6. Neuronal Calcium Channels

Jeremy Gilmore, Colin Dell, David Bowman and David Lodge
Lilly Research Centre Ltd., Eli Lilly and Co., Erl Wood Manor,
Windlesham, Surrey GU20 6PH, U.K.

Introduction - Voltage-dependent calcium channels (VDCC) belong to a class of proteins that mediate ionic movements across excitable cell membranes in response to changes in membrane potential. The proposed topology of the pore-forming element is shared with that of voltage-dependent sodium and potassium channels. Thus in VDCCs an α_1 subunit consists of four domains, each of which has six trans-membrane spanning segments (TM). The fourth TM of each domain has positively charged residues at every third amino acid of its α helix and is thought to be the voltage-sensor. Changes in the conformation of this segment and the associated protein substructures as a result of cell membrane depolarisation allow the permeation of calcium ions through the pore. The pore is largely formed by the four extracellular loops between TM5 and TM6 of each domain, each loop dipping into the membrane to line the mouth of the channel made by apposition of the four domains. The α1 pore-forming protein is co-assembled into the membrane with at least two other subunits, named $\alpha_2\delta$ and β (Figure 1).

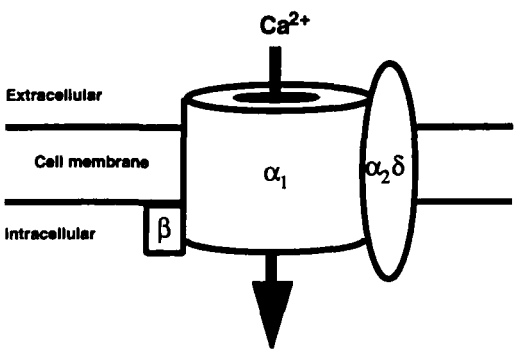


Fig. 1. Subunit composition of neuronal calcium channel

Normally the extracellular calcium concentration is about 2 mM while the intracellular concentration is <1 μM and hence on opening calcium ions permeate into the cell. This entry of calcium triggers many processes in cells including the activation of calcium-dependent enzymes and second messengers, excitation-contraction coupling in smooth and cardiac muscle cells, excitation-secretion coupling in nerve terminals and transmission of action potentials along nerve cells. It is not surprising then that there are a variety of different types of VDCC mediating these events. Recent comprehensive reviews have described characterisation of VDCCs using biophysics, pharmacology and molecular biology techniques (1,2,3,4,5,6).

The subtypes of VDCC are commonly referred to as L, N, P, Q, R and T (Table 1). Only small changes in membrane potential are required to activate T-type channels and hence these are named low voltage activated (LVA). The remainder, requiring greater depolarisation for activation, are known as high voltage activated (HVA) channels. Members of the L channel family are dihydropyridine (DHP) -sensitive and extensive reviews on the associated medicinal chemistry and physiology are available (7, 8, 9).

In the present review we shall concentrate on neuronal VDCCs of the N and P-Q families, because of their likely relevance to neurotransmitter release (see below).

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Pharmacology - The early subdivision of VDCCs into L-, T- and N-type channels was based largely on biophysical characteristics, although both T and N types were resistant to dihydropyridines (10). It soon became apparent, however, that N-type channels were uniquely blocked by nanomolar concentrations of a peptide toxin, ω-CgTx-GVIA, found in the venom of a fish hunting mollusc, *Conus geographus* (11, 12). Inhibition of a calcium channel by this toxin is widely accepted as being indicative of N-type pharmacology. The inhibition is practically irreversible whereas that by another selective toxin, ω-CmTx-MVIIA from *Conus magus*, is readily reversible (13).

Channel type	Activation	α_1 Subunit	Toxin sensitivity
T	LVA	?	sFTX 3.3
L	HVA	$\alpha_{1C/D}$	Calciceptin
N	HVA	α_{1B}	ω-CgTx-GVIA ω-CmTx-MVIIA
P	HVA	α_{1A}?	ω-Aga-IVA
Q	HVA	α_{1A}?	ω-CmTx-MVIIC
R	HVA	α_{1E}	-

Table 1. VDCC subtypes

P-type channels were first described in cerebellar Purkinje cells where they contribute about 90% of the somal calcium current (14). Such channels are blocked by nanomolar concentrations of ω-Aga-IVA, a peptide toxin from the venom of the American funnel web spider, *Agelenopsis aperta* (15).

Measuring calcium currents from cell bodies of many different neurones throughout the CNS, however, revealed there were residual currents even in the presence of L-, N- and P-type channel blockers. Part of these currents, also blocked by micromolar concentrations of ω-Aga-IVA, was shown to be sensitive to 100 nM concentrations of ω-CmTx-MVIIC, and termed the Q current (16). Unfortunately, in terms of pharmacological specificity, this toxin also blocks P-type channels with submicromolar potency, blocks at least some N-type channels with nanomolar potency and, at subnanomolar concentrations, binds to neuronal membranes and reduces hippocampal norepinephrine release (17).

The residual current in the presence of all the above organic blockers was named the R fraction. In some cells at least, this pharmacologically resistant calcium current was found to be rapidly inactivating (15).

Molecular Biology of Ion Channels - There are known to be at least six α_1 genes coding for the pore-forming proteins of HVA VDCCs (18). These are named α_{1S}, α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E} (19). No gene coding for T-type LVA VDCCs has yet been cloned. The α_{1S}, α_{1C} and α_{1D} genes code for dihydropyridine-sensitive L channels and are found in skeletal muscle, heart and brain, and brain tissue respectively. The α_{1A} (20), α_{1B} (21) and α_{1E} (22, 23) genes are thought to code for P/Q-, N- and R-type VDCC families respectively and are found mainly in neuronal tissue. Pharmacological heterogeneity within these families (see below) may result from post-transcriptional and -translational modifications, from expression of other as yet unknown genes, and/or from different subunit combinations. Thus in addition to the α_1 genes of neuronal VDCCs, $\alpha_2\delta$ and β genes have also been cloned and expressed. Only one variant, $\alpha_{2b}\delta$, of the $\alpha_2\delta$ subunit has been elucidated in the CNS whereas by contrast there are four genes, β_1-β_4, coding for the β subunits. At this stage, the β subunit

appears more influential in modifying the biophysical (eg. rates of activation and inactivation) rather than the pharmacological properties of the α_1 pore-forming element (24). Permutations of subunit combinations and splice variants of α_1, $\alpha_2\delta$ and β subunits add to the possible complexity of the neuronal VDCC families.

Expression of cloned subunits in oocytes and mammalian cell lines is allowing comparison of the biophysics and pharmacologies of native and engineered channels. All cloned α_1 subunits give rise to HVA channels. Expression of α_{1A} (18) and α_{1B} (19) gives rise to slowly inactivating channels whereas those expressed by α_{1E} (21) genes are rapidly inactivating and more akin to R channels. Like R channels, α_{1E} expression leads to VDCCs that are insensitive to ω-CgTx-GVIA, ω-CmTx-MVIIC and ω-Aga-IVA (21). α_{1B} and α_{1A} give rise to VDCCs that are respectively blocked by ω-CgTx-GVIA and ω-Aga-IVA which suggests an equivalence with N- and P-type native channels. This appears to be an acceptable biophysical and pharmacological correlation of α_{1B} and N-type VDCCs. The ω-Aga-IVA sensitivity of the engineered α_{1A} channels presently described (18) is, however, considerably less than that observed in native P-type VDCCs where only low nanomolar concentrations are required for block (14). Indeed, this α_{1A} pharmacology is more akin to that of Q-type VDCCs (5). Hence it may be more appropriate to think of the α_{1A} gene as coding for the P-Q family rather than for P alone. Further work, using combinations with different β subunits and with splice variants of α_1 and β subunits, may help with this conundrum.

Neurotransmitter Release - One of the intriguing hypotheses raised by neuronal VDCC diversity is that neurotransmitter release may be differentially modified by subtype selective VDCC modulators. If confirmed, this hypothesis could generate a whole new therapeutic approach to neurological and psychiatric diseases. The first requisite for such a hypothesis is that VDCCs mediating transmitter release are not pharmacologically identical, and this indeed seems to be the case. Potassium-stimulated norepinephrine release is most sensitive to ω-CgTx-GVIA (25) whereas glutamate (26, 27) and GABA (20) release is most sensitive to ω-Aga-IVA. Such differential sensitivity is most noticeable at low levels of KCl, higher levels apparently recruiting other channel types, presumably at the same terminals, into the release process. To block this higher level of release, combinations of toxins may be required (28). Similar results are found in electrophysiological studies of synaptic excitation. Thus, at the the glutamatergic Schaffer collateral to CA1 synapse, 100 nM concentrations of ω-Aga-IVA or ω-CmTx-MVIIC produce a near complete block, whereas ω-CgTx-GVIA produces only a partial block (29). Such studies, and parallel ones using immunocytochemical markers to locate VDCC subtypes at nerve terminals, are still in their infancy but may have profound pharmacologic and therapeutic ramifications.

LIGANDS FOR NEURONAL CALCIUM CHANNELS

Invertebrate Toxins - Venoms from a diverse range of invertebrate species have provided peptide neurotoxins that act on neuronal VDCCs (30). As has been described above, selective toxins derived from molluscs and spiders have allowed the pharmacological definition of subclasses of neuronal calcium channels (Figure 2).

The ω-conotoxins, isolated from the venom of fish-hunting snails of the genus *Conus*, are globular peptides of 24-29 amino acids, containing a conserved pattern of three disulfide bridges (31). ω-CgTx-GVIA (from *Conus geographus*) and ω-CmTx-MVIIA (from *Conus magus*) have defined the N-type VDCC, while ω-CmTx-MVIIC at appropriate concentrations is selective for Q-type channels. ω-CmTx-MVIID appears to be selective for non-N-type channels (32).

ω-Conotoxins:

ω-CgTx-GVIA $C^1KSOGSSC^2SOTSYNC^3C^1RSC^2NOYTKRC^3Y\text{-}NH_2$

ω-CmTx-MVIIA $C^1KGKGAKC^2SRLMYDC^3C^1TGSC^2RSGKC^3\text{-}NH_2$

ω-CmTx-MVIIC $C^1KGKGAPC^2RKTMYDC^3C^1SGSC^2GRRGKC^3\text{-}NH_2$

ω-Agatoxins:

ω-AgaIVA KKKC[1]IAKDYGRC[2]KWGGTPC[3]C[1]RGRGC[2]IC[4]SIM-GTNC[4]EC[3]KPRLIMEGLGLA

Fig 2. Structures of toxins most widely employed in the toxityping of VDCCs. (O = 4-hydroxyproline. Superscripts denote pattern of cystine disulfide bridges.)

There have been several reports of the solution structure of ω-CgTx-GVIA (33,34,35) derived from 2-D NMR studies, using hybrid distance geometry and restrained molecular dynamics approaches. The secondary structure contains an antiparallel, triple-stranded β-sheet containing a β-hairpin and 3 tight turns. The 3-D structure is amphiphilic with a positively charged hydrophilic side and a small hydrophobic region on the opposite face - the residues thought to be important for binding and function are located on the hydrophilic face. This β-sheet motif also appears as a common structural feature in several other unrelated toxins and enzyme inhibitors (36). The solution structure of ω-CmTx-MVIIC has also been determined by ^{1}H-NMR and simulated annealing calculations (37). Lys^{25} is buried within the molecule and relatively inflexible, so that of the four Lys residues present in the peptide, it is the least reactive towards biotinylation, and such derivatisation has the least influence on biological activity.

Chemical modification or site specific mutation of the ω-conotoxins has led to an understanding of the residues key to their binding to VDCCs. Reaction of ω-CgTx-GVIA with nonsaturating concentrations of acetic anhydride showed that acetylation of the amino terminus on Cys^1 gave greater loss of potency than acetylation of the ε-amino groups of Lys^2 or Lys^{24} (38). Systematic replacement of the four basic residues of ω-CgTx-GVIA with alanine demonstrated that Arg^{17}, Lys^{24} and Arg^{25} were not important for binding, while Lys^2 replacement gave only a modest 40-fold decrease in potency compared to the native toxin (39). However, replacement of Tyr^{13} in either ω-CgTx-GVIA or ω-CgTm-MVIIA with alanine gave approximately 500-fold reduction in potency (40).

ω-CgTx-GVIA (10 and 100 μM by continuous perfusion) inhibited the release of dopamine in the striatum of spontaneously hypertensive rats before and during transient cerebral ischemia during an *in vivo* dialysis study (41). ω-CgTm-MVIIA (SNX-111) gave protection of the pyramidal neurons of the CA1 region of the hippocampus in the rat four-vessel occlusion model of transient global ischemia, even when given as a single bolus injection (5mg/kg) (42). The toxin by i.v. infusion also reduced infarct size in rats subjected to 2h. of middle cerebral artery occlusion (MCAO) (43). The compound has now advanced into clinical trials for ischemia (44). SNX-111 given intrathecally also inhibited formalin-induced nociception (ED_{50} = 0.003 nmol) (45).

ω-Agatoxins, purified from the venom of the American funnel web spider *Agelenopsis aperta*, are globular peptides of 48 to 76 amino acids, with at least 4 disulfide bridges and a rigid core similar to that of the ω-conotoxins (4). ω-Aga-IVA has proved invaluable in defining P-type calcium channels (14) and their association with the release of glutamate (46). ω-Aga-IVB (ω-Aga-TK), which is 12 times more abundant than Aga-IVA in the spider venom, has also been found to be potent and selective at P-channels (47). ω-Agatoxins IA, IIA and IIIA show a more complex pattern of channel selectivity in synaptosomal preparations, explained either by the presence of distinct channel subtypes or alteration of the kinetics of a homogeneous channel population (48, 49).

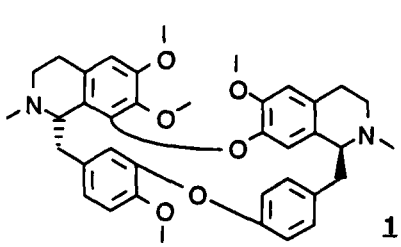
3-D structures of ω-agatoxins IVA and IVB have been proposed, possessing a small 3-stranded antiparallel β-sheet and 4 loop regions. Three conserved arginines may be important for specific interaction with P-type VDCCs, and the N-terminal amino acids influence the kinetics of binding (50).

ω-Aga-IVA has been synthesised by conventional solution procedures, together with its N-terminal biotinylated and tripeptide truncated forms. The N-terminus Lys^1-Lys^2-Lys^3 was not important in protein folding or for activity, but modification of Trp^{14} gave significantly reduced activity (51). Synthesis of ω-Aga-IVB revealed that Ser^{46} in the natural toxin possessed the D-configuration, with the ω-[L-Ser^{46}]Aga-IVB being 80-90 fold less potent than the native peptide at blocking P-type channels (52).

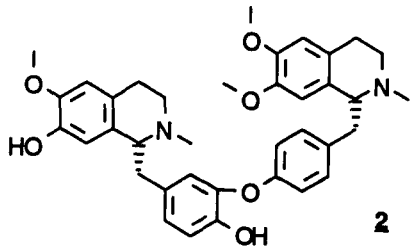
The venom from *Agelenopsis* has also yielded a low-molecular weight toxin FTX, reported to be active at P-type VDCCs (53). Two polyamine structures have so far been proposed for this toxin, but in both cases the synthetic materials have possessed physical and biological characteristics inconsistent with those of the natural product (54, 55).

A number of other spider venoms have been shown to interact with VDCCs. The crude venom of *Plectreurys tristis* inhibits both N- and non-L/non-N-channels, but has no effect on L-channels (56); while that of the funnel web spider *Hololena curta* blocks the DHP/ω-CgTx-GVIA insensitive component of rat synaptosome Ca^{2+} influx (57). ω-Grammatoxin SIA (from the tarantula spider, *Grammostola spatulata*), a 36 amino acid peptide, blocks all of the $^{45}Ca^{2+}$ influx into rat synaptosomes (IC_{50} = 0.2 μM) (58) and whole-cell Ca^{2+} current in rat dorsal root ganglia (DRG) neurones (59). Calcicludine (from the green mamba, *Dendroaspis angusticeps*), a 60 amino acid polypeptide, blocks most HVA VDCCs in the 10-100 nM range, although it is most potent at L-type channels ($K_{0.5}$ = 0.2 nM in rat cerebellar granule cells) (60).

Other Natural Products - Bis-benzylisoquinoline alkaloids, isolated from traditional Chinese medicinal herbs, have shown activity as VDCC blockers. Tetrandrine **1** (from *Radix stefania tetrandra*) is a non-specific blocker of LVA and HVA channels in cultured spinal cord neurones of foetal mice (K_d = 8 μM) (61). (*R,R*)-(-)-Daurisoline **2** (from *Menispermum dauricum*) was tentatively proposed to act on N-type VDCCs and inhibited glutamate release *in vivo* after i.v. administration (62). More recently, it has been shown to inhibit ω-Aga-IVA-sensitive Ba^{2+} currents in rat cerebellar Purkinje cells (IC_{50} = 35 μM) while not affecting agatoxin-insensitive currents in cerebellar granule cells at 100 μM (63).



1

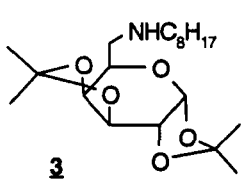


2

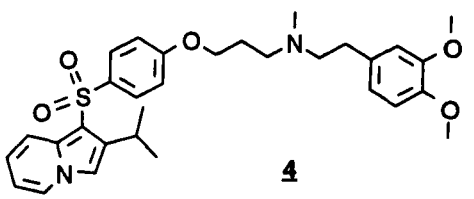
A number of aminoglycosides interact with neuronal VDCCs at high micromolar concentrations (64). Neomycin is a non-selective inhibitor of neuronal responses mediated by L-, N- and non-L/non-N-type channels with IC_{50} = 90-400 μM (65). The synthetic aminoglycoside 8RN-DAGal **3** shows a voltage-dependent block of P-channels expressed in *Xenopus* oocytes (70% block at 15 μM) (66).

Synthetic Ligands - A number of L-channel antagonists have also been attributed neuronal non-L-channel activities. Verapamil, emopamil and diltiazem inhibit KCl-

evoked [^{3}H]-D-aspartate release from rat hippocampal slices (67, 68), a process insensitive to nitrendipine and ω-CgTx-GVIA, implying a neuronal non-L-non-N-type VDCC. (*S*)-Emopamil shows activity in animal models of cerebral ischemia, one particular observation being the attenuation of the post-ischemic increase in hippocampal extracellular glutamate concentration (69). Controversy exists however over whether the neuroprotective effect is at least in part due to the potent 5HT-2 receptor blockade exhibited by emopamil (70). Fantofarone (SR 33557, **4**), a compound which binds to the α_1 subunit of the L-type Ca^{2+} channel, also has N- and P-type VDCC blocking properties as measured by whole cell patch clamping in chick DRG and rat cerebellar Purkinje cell preparations respectively (71). The dihydropyridine (±)-BAY K 8644 blocks P-type VDCCs (72).



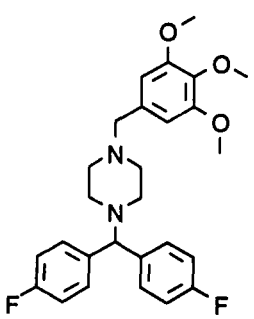
3



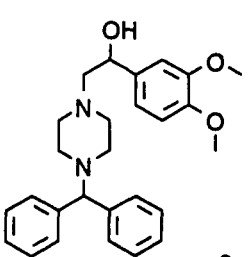
4

A number of classical neuroleptics, including chlorpromazine, thioridazine, fluphenazine, fluspirilene, pimozide, haloperidol and spiperone, have been shown to possess N- and P-channel blocking properties by the inhibition of Ca^{2+} currents in rat sympathetic neurons and rat cerebellar Purkinje neurons measured by whole cell patch clamp (73). Fluspirilene was the most potent (IC_{50} values of 6 μM and 2 μM for N- and P-channel blockade respectively). The tricyclic antidepressants imipramine, desipramine, amitryptyline and clomipramine inhibit depolarisation-evoked $^{45}Ca^{2+}$ uptake into rat brain cortical synaptosomes, all with IC_{50} values in the region of 30 μM (74).

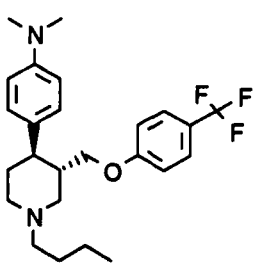
Flunarizine inhibits K^+-evoked Ca^{2+} influx into gerbil cortical synaptosomes, a preparation insensitive to nimodipine and nifedipine but sensitive to ω-CgTx-GVIA (75). The related piperazine KB-2796 (**5**) inhibits the non-L-type HVA Ca^{2+} current in rat hippocampal CA1 neurons (IC_{50} 4 μM , *cf.* flunarizine 3.1 μM) (76). Another piperazine NC-1100 (**6**) is less potent in this preparation (77).



5



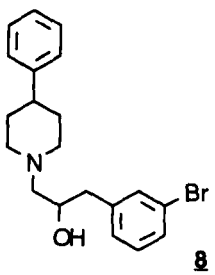
6



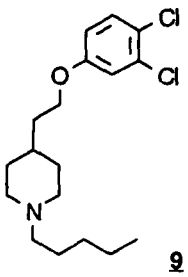
7

A variety of piperidines also possess non-L-type VDCC blocking properties. NNC 09-0026 (**7**) inhibits K^+-stimulated $^{45}Ca^{2+}$ uptake into rat cortical synaptosomes with an IC_{50} of 13 μM (78). Inhibition also occurs in the presence of L- and N-channel blockade indicating activity at multiple VDCC types. The piperidine (**8**) is one of a number of related compounds found to block L- and N-channels in rat DRG (79). SB 201823-A (**9**) is also active in such neuronal preparations (80). R56865 (**10**) is

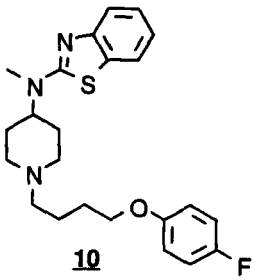
reportedly active on P-type VDCCs (81). Interestingly, NNC 09-0026, SB 201823-A and R56865 are all active in animal models of cerebral ischemia (78, 80, 81).



8

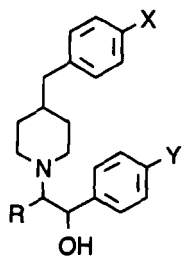


9

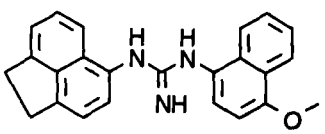


10

The structurally similar NMDA receptor antagonists eliprodil (11) and ifenprodil (12) also have activity against neuronal Ca^{2+} channels. Eliprodil blocks the inward Ba^{2+} current in rat cortical neurons (part L-, part N-type) and in rat Purkinje cells (P-type) as measured by whole cell patch clamp with IC_{50} values in the 1.5 μM range (82). In cultured rat hippocampal pyramidal neurons, ifenprodil was able to block the dihydropyridine insensitive K^+-evoked Ca^{2+} influx with an IC_{50} of 13 μM (83).



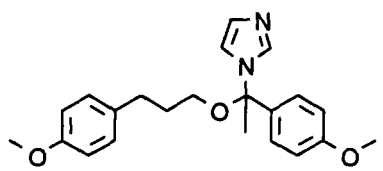
11 (R=H, X=F, Y=Cl)
12 (R=Me, X=H, Y=OH)



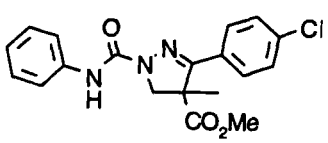
13

The diarylguanidine CNS 1237 (13) inhibits K^+-evoked $^{45}Ca^{2+}$ uptake into rat cortical synaptosomes and also inhibits the K^+-stimulated Ca^{2+}-dependent glutamate release in rat brain synaptosomes (84). The latter process is reportedly sensitive to P/Q type VDCC antagonists. CNS 1237 is active in the rat MCAO model of ischemia.

Other miscellaneous compounds reported to possess neuronal non-L-type VDCC activities include the nootropic agent aniracetam showing 30% block of N-type channels at 500 μM (85), substituted benzimidazoles active on L-, N- and P-type VDCCs (86), simple aliphatic alcohols active on the P-type channel (87), SK&F 96365 (14) a receptor-mediated Ca^{2+} entry blocker (88), RH-5529 (15) a Na^+ channel blocker (89) and the anti-diarrheal drug loperamide (90).



14



15

Conclusions - The understanding of the biology of neuronal calcium channels has developed significantly over the last five years with the recognition of at least six

subtypes of VDCCs found in the CNS. Molecular biology studies are revealing the subunit composition of these individual subtypes and allowing the development of cloned cell lines expressing single populations of neuronal VDCCs. To date, potent and selective agents for specific channel subtypes are restricted to the invertebrate polypeptide toxins, but a knowledge of the solution structures of these macromolecules may aid in the development of low molecular weight ligands. *In vivo* studies with ω-CmTx-MVIIA, an N-channel specific toxin, have demonstrated its efficacy in models of ischemia. With an increasing understanding of the differential localisation of VDCC subtypes at nerve terminals, there is an exciting prospect for the development of novel CNS therapeutic agents which intervene in the neurotransmitter release process *via* interaction with neuronal voltage-dependent calcium channels.

References

1. R.W. Tsien, D. Lipscombe, D.V. Madison, K.R. Bley and A. P. Fox, Trends Neurosci., 11, 431 (1988).
2. B.P. Bean, Ann. Rev. Physiol., 51, 367 (1989).
3. R.J. Miller and A.P. Fox., in 'Intracellular Calcium Regulation', F.Bronner, Ed., Wiley-Liss, New York, 1990, p. 97.
4. B.M. Olivera, G.P. Miljanich, J. Ramachandran and M.E. Adams, Ann. Rev. Biochem., 63, 823, (1994)
5. D.B. Wheeler, W.A. Sather, A. Randall and R.W. Tsien. in 'Molecular and Cellular Mechanisms of Neurotransmitter Release', L. Stjarne, P. Greengard, S. Grilner, T. Hokfelt and D. Ottoson, Ed., Raven Press, New York, 1994, p. 155.
6. K. Dunlap, J.I. Luebke and T.J. Turner, Trends Neurosci., 18, 89 (1995)
7. R.A. Janis, P.J. Silver and D.J. Triggle, Adv. Drug Res., 16, 309 (1987)
8. G. Varadi, Y. Mori, G. Mikala and A. Schwartz, Trends Pharmacol. Sci., 16, 43 (1995)
9. F. Hofmann, M. Biel and V. Flockerzi, Ann. Rev. Neurosci., 17, 399 (1994).
10. M.C. Nowycky, A.P. Fox and R.W. Tsien, Nature, 316, 440 (1985).
11. L.J. Cruz and B.M. Olivera, J. Biol. Chem., 261, 6230 (1986).
12. A.P. Fox, M.C. Nowycky and R.W. Tsien, J. Physiol., 394, 149 (1987).
13. C.J. Grantham, D. Bowman,C.P. Bath and D. Bleakman, Neuropharmacology, 33, 255 (1994).
14. R. Llinas, M. Sugimori, J-W. Lin and B. Cherksey, Proc. Natl. Acad. Sci. USA, 86, 1689 (1989).
15. I.M. Mintz, V.J. Venema, K.M. Swiderek, T.D. Lee, B.P. Bean and M.E. Adams, Nature, 355, 827 (1992).
16. A.W. Randall, B. Wetland, F. Schweizer, G. Miljanich, M.E. Adams and R.W. Tsien, Soc. Neurosci. Abs., 19 1478 (1993)
17. D.R. Hillyard, V.D. Monje, I.M. Mintz, B.P. Bean, L. Nadasidi, J. Ramachandran, G. Miljanich, A. Azimi-Zoonooz, J.M. McIntosh, L.J. Cruz, J.S. Imperial and B.M. Olivera, Neuron, 9, 69 (1992)
18. J-F. Zhang, A.D. Randall, P.T. Ellinor, W.A. Horne, W.A. Sather, T. Tanabe, T.L. Schwarz, and R.W. Tsien, Neuropharmacology, 32, 1075 (1993).
19. L. Birnbaumer, K.P. Campbell, W.A. Catterall, M.M. Harpold, F. Hofmann, W.A. Horne, Y. Mori, A. Schwartz, T.P. Snutch, T. Tanabe and R.W. Tsien, Neuron, 13, 505 (1994).
20. W.A. Salter, T. Tanabe, Y. Mori, M.E. Adams and R.W. Tsien, Neuron, 11, 291 (1993).
21. M.E. Williams, P.F. Brust, D.H. Feldman, S. Patthi, S. Simerson, A. Maroufi, A.F. McCue, G. Velicelebi, S.B. Ellis and M.M. Harpold, Science, 257, 389 (1992).
22. T.W. Soong, A. Stea, C.D. Hodson, S.J. Dubel, S.R. Vincent and T.P. Snutch, Science, 260, 1133 (1993).
23. M.E. Williams, D.H.Feldman, A.F. McCue, R. Brenner, G. Velicelebi, S. Ellis and M.M. Harpold, Neuron, 8, 71 (1992).
24. Y. Mori, T. Fredrich, M-S. Kim. A. Mikami, J. Nakai, P. Roth, E. Bosse, F. Hoffmann, V. Flockerzi, T. Furuichi, K. Mikoshiba, K. Imoto, T. Tanabe and S. Numa, Nature, 350, 398 (1991).
25. S. Gaur, R. Newcombe, B. Rivnay, J.R. Bell, D. Yamashiro, J. Ramachandran and G.P. Miljanich, Neuropharmacology, 33, 1211 (1994)
26. J.L. Luebke, K. Dunlap and T.J. Turner, Neuron, 11, 895 (1993)

27. T. Takahashi and A. Momiyama, Nature, 366, 156 (1993).
28. T.J. Turner, M.E. Adams and K. Dunlap, Proc. Natl. Acad. Sci. USA, 90, 9518 (1993).
29. D.B. Wheeler, A. Randall and R.W. Tsien, Science, 264, 107 (1994).
30. N.A. Saccomano and M.K. Ahlijinian, Drug Dev. Res., 33, 319 (1994).
31. R.A. Meyers, L.J. Cruz, J.E. Rivier and B.M. Olivera, Chem. Rev., 93, 1923 (1993).
32. V.D. Monje, J.A. Haack, S.R. Naisbitt, G. Miljanich, J. Ramachandran, L. Nasdasdi, B.M. Olivera, D.R. Hillyard and W.R. Gray, Neuropharmacol., 32, 1141 (1993).
33. J.H. Davies, E.K. Bradley, G.P. Miljanich, L. Nadasdi, J. Ramachandran and V.J. Basus, Biochem., 32, 7396 (1993).
34. J.J. Skalicky, W.J. Metzler, D.J. Ciesla, A. Galdes and A. Paıdi, Prot. Sci., 2, 1591 (1993).
35. P.K. Pallaghy, B.M. Duggan, M.W. Pennington and R.S. Norton, J. Mol. Biol., 234, 405 (1993).
36. P.K. Pallaghy, K.J. Nielsen, D.J. Craik and R.S. Norton, Prot. Sci., 3, 1833 (1994).
37. N. Nemoto, S. Kubo, T. Yoshida, N. Chino, T. Kimura, S. Sakakibara, Y. Kyogoku and Y. Kobayashi, Biochem. Biophys. Res. Commun., 207, 695 (1995).
38. R.A. Lampe, M.M.S. Lo, R.A. Keith, M.B. Horn, M.W. McLane, J.L. Herman and R.C. Spreen, Biochem., 32, 3255 (1993).
39. K. Sato, N.-G. Park, T. Kohno, T. Maeda, J.I. Kim, R. Kato and M. Takahashi, Biochem. Biophys. Res. Commun., 194, 1292 (1993).
40. J.I. Kim, M. Takahashi, A. Ohtake, A. Wakamiya and K. Sato, Biochem. Biophys. Res. Commun., 206, 449 (1995).
41. H. Ooboshi, S. Sadoshima, H. Yao, T. Nakahara, H. Uchimura and M. Fujishima, J. Neurochem., 58, 298 (1992).
42. K. Valentino, R. Newcomb, T. Gadbois, T. Singh, S. Bowersox, S. Bitner, A. Justice, D. Yamashiro, B.B. Hoffman, R. Ciaranello, G. Miljanich and J. Ramachandran, Proc. Natl. Acad. Sci. USA, 90, 7894 (1993).
43. Q. Zhao, M.-L. Smith and B.K. Siesjo, Acta Physiol. Scand., 150, 459 (1994).
44. S.S. Bowersox and R.R. Luther, Drugs Future, 19, 128 (1994).
45. A.B. Malmberg and T.L. Yaksh, J. Neurosci., 14, 4882 (1994).
46. T.J. Turner, M.E. Adams and K. Dunlap, Science, 258, 310 (1992).
47 T. Teramoto, M. Kuwada, T. Niidome, K. Sawada, Y. Nishizawa and K. Katayama, Biochem. Biophys. Res. Commun., 196, 134 (1993).
48. V.J. Venema, K.M. Swiderek, T.D. Lee, G.M. Hathaway and M.E. Adams, J. Biol. Chem., 267, 2610 (1992).
49. J.M. Pocock, V.J. Venema and M.E. Adams, Neurochem. Int., 20, 263 (1992).
50. M.D. Reily, K.E. Holub, W.R. Gray, T.M. Norris and M.E. Adams, Structural Biology, 1, 853 (1994).
51. H. Nishio, K.Y. Kumagaye, S. Kubo, Y. Chen, A. Momiyama, T. Takahashi, T. Kimura and S. Sakakibara, Biochem. Biophys. Res. Commun., 196, 1447 (1993).
52. M. Kuwada, T. Teramoto, K.Y. Kumagaye, K. Nakajima, T. Watanabe, T. Kawai, Y. Kawakami, T. Niidome, K. Sawada, Y. Nishizawa and K. Katayama, Mol. Pharmacol., 46, 587 (1994).
53. B.D. Cherksey, M. Sugimori and R.R. Llinas, Ann. N. Y. Acad. Sci., 636, 30 (1991).
54. R.H. Scott, M.I. Sweeney, E.M. Kobrinsky, H.A. Pearson, G.H. Timms, I.A. Pullar, S. Wedley and A.C. Dolphin, Br. J. Pharmacol., 106, 199 (1992).
55. I.S. Blagbrough and E. Moya, Tet. Lett., 35, 2057 (1994).
56. P.M. Lundy and R. Frew, Toxicon, 31, 1249 (1993).
57. P.M. Lundy, A. Hong and R. Frew, Eur. J. Pharmacol., Mol. Pharm. Sect., 225, 51 (1992).
58. R.A. Lampe, P.A. DeFeo, M.D. Davison, J. Young, J.L. Herman, R.C. Spreen, M.B. Horn, T.J. Mangano and R.A. Keith, Mol. Pharmacol., 44, 451 (1993).
59. T.M. Piser, R.A. Lampe, R.A. Keith and S.A. Thayer, Pflugers Arch., 426, 214 (1994).
60. H. Schweitz, C. Heurteaux, P. Bois, D. Moinier, G. Romey and M. Lazdunski, Proc. Natl. Acad. Sci. USA, 91, 678 (1994).
61. U. Bickmeyer and H. Wiegand, NeuroReport, 4, 938 (1993).
62. Y. Ming Lu and G.Q. Liu, Br. J. Pharmacol., 101, 45 (1990).
63. Y. Ming Lu, W. Frostl, J. Dreesen and T. Knopfel, NeuroReport, 5, 1489 (1994).
64. T.D. Parsons, A.L. Obaid and B.M. Salzberg, J. Gen. Physiol., 99, 491 (1992).
65. R.A. Keith, T.J. Mangano, P.A. DeFeo, M.B. Horn and A.I. Salama, J. Mol. Neurosci., 3, 147 (1992).

66. F. Fournier, G. Charpentier, A. Lahyani, J. Bruner, G. Czternasty, D. Marlot, G. Ronco, P. Villa and G. Brule, J. Pharm. Belg., 48, 445 (1993).
67. R.A. Keith, T.J. Mangano, P.A. DeFeo, G.E. Ernst and E.J. Warawa, Br. J. Pharmacol., 113, 379 (1994).
68. T.J. Mangano, J. Patel, A.I. Salama and R.A. Keith, Eur. J. Pharmacol., 192, 9 (1991).
69. M. Matsumoto, M.S. Scheller, M.H. Zornow and M.A.P. Stmat, Stroke, 24, 1228 (1993).
70. F.V. Defeudis, Trends Pharmacol. Sci., 10, 215 (1989).
71. G. Romey and M. Lazdunski, J. Pharmacol. Exp. Ther., 271, 1348 (1994).
72. M.M. Usowicz, M. Sugimori, B. Cherksey and R. Llinas, Neuron, 9, 1185 (1992).
73. D.W.Y. Sah and B.P. Bean, Mol. Pharmacol., 45, 84 (1994).
74. P.-A. Lavoie, G. Beauchamp and R. Elie, Can. J. Physiol. Pharmacol., 68, 1414 (1990).
75. S.L. Cohan, D.J. Redmond, M. Chen, D. Wilson and P. Cyr, J. Cereb. Blood Flow Metab., 13, 947 (1993).
76. N. Akaike, H. Ishibashi, H. Hara, Y. Oyama and T. Ueha, Brain Res., 619, 263 (1993)
77. N. Miyake, M. Wakamori and N. Akaike, Brain Res. 598, 215 (1992).
78. F.C. Barone, W.J. Price, P. Jakobsen, M.J. Sheardown and G. Feuerstein, Pharmacol. Biochem. Behav., 48, 77 (1994).
79. A.S. Sipahimalani, J.L. Werth, R.H. Michelson, A.K. Dutta, S.M.N. Efange and S.A. Thayer, Biochem. Pharmacol., 44, 2039 (1992).
80. C.D. Benham, T.H. Brown, D.G. Cooper, M.L. Evans, M.H. Harries, H.J. Herdon, J.E. Meakin, K.L. Murkitt, S.R. Patel, J.C. Roberts, A.L. Rothaul, S.J. Smith, N. Wood and A.J. Hunter, Neuropharmacology, 32, 1249 (1993).
81. V.A. Panchenko, O.A. Krishtal, F.Tegtmeier and A.Y. Tsyndrenko, Neuroscience, 54, 587 (1993).
82. B. Biton, P. Granger, A. Carreau, H. Depoortere, B. Scatton and P. Avenet, Eur. J. Pharmacol., 257, 297 (1994).
83. J. Church, E.J. Fletcher, K. Baxter and J.F. MacDonald, Br. J. Pharmacol., 113, 499 (1994).
84. N.L. Reddy, D. Daly, G.J. Durant, S.M. Goldin, L.-Y. Hu and K. Subbarao, Soc. Neurosci. Abs., 20, 70 (1994).
85. H. Koike, H. Saito and N. Matsuki, Japan. J. Pharmacol., 61, 277 (1993).
86. O. Axelsson, D. Peters and E.O. Nielsen, Eur. Pat. 604353 (1994).
87. M.H. Hawthorn, J.N. Ferrante, Y.W. Kwon, A. Rutledge, E. Luchowski, R. Bangalore and D.J. Triggle, Eur. J. Pharmacol., 229, 143 (1992).
88. E. Tachikawa, S. Takahashi, K. Mizuma, Y. Kondo, T. Kashimoto and E. Takahashi, Neurosci. Lett., 177, 155 (1994).
89. A. Zhang and R.A. Nicholson, Pestic. Biochem. Physiol., 45, 242 (1993).
90. J. Church, E.J. Fletcher, K. Abdel-Hamid and J.F. MacDonald, Mol. Pharmacol., 45, 747 (1994).

SECTION II. CARDIOVASCULAR AND PULMONARY DISEASES

Editor: Annette M. Doherty
Parke-Davis Pharmaceutical Research, Ann Arbor, MI 48105

Chapter 7. Vascular Proliferative Disease

Larry A. Denner and Richard A.F. Dixon
Texas Biotechnology Corporation
Houston, TX 77030

Introduction - Vascular proliferative diseases arise by many diverse mechanisms. The most common initial form is atherosclerosis, a vascular occlusive event that occurs over the course of many years or decades as a consequence of abnormal cholesterol deposition in vessel walls. Treatments are also very diverse and while initially often successful, induce a secondary narrowing called restenosis. The mechanisms of this disease process are quite different from atherosclerosis. A variety of drugs have been discovered and developed that inhibit the underlying cellular processes in restenosis and the manifestation of this disease in animal models. However the success rate for reduction of clinical restenosis in humans has been minimal at best. This leaves open the opportunities for novel strategies to impact on the quality of life for this large and growing patient population.

VASCULAR PHYSIOLOGY IN HEALTH AND DISEASE

Normal Vessel Structure and Function - Normal blood vessels are fairly simple in cellular constitution (1). Endothelial cells (ECs) line the lumen and provide a selective barrier between the blood and the underlying vessel wall. These ECs are separated from smooth muscle cells (SMCs) by a thick layer of elastin, the internal elastic lamina (IEL). SMCs comprise the media and are primarily responsible for vessel contractility and the maintenance of blood flow initiated by contraction of the heart. Variable numbers of individual layers of SMCs are separated by thinner layers of elastin. Large muscular arteries may have 5-10 layers of SMCs while elastic arteries and veins may have as few as 1 layer. The outermost layer of SMCs is limited by the external elastic lamina which is covered by adventitial connective tissue composed predominantly of fibroblasts and a rather rich mixture of matrix molecules, most notably collagen. The adventitia often has smaller arterioles, venuoles, nerve bundles, limp ducts, and vasa vasorum.

The normal primary function of blood vessels is to carry nutrients and oxygen to vascular capillary beds and to carry metabolic products and carbon dioxide for removal from the body. Natural defense systems have evolved to insure control of these functions. These include platelet adhesion at the luminal surface of injured vessel walls which induce the formation of thrombi that lead to clot formation and prevention of bleeding into the extravascular space. In the case of extensive insults to the vessel wall, inflammatory cells will also accumulate to aid in vascular repair.

Pathophysiology - Under certain conditions these normal protective mechanisms are overstimulated and cause vascular diseases, the most common being atherosclerosis (2-4). Although the detailed etiology of atherosclerosis is very complex, some typical observations have been made. Abnormal deposition or accumulation of cholesterol in the vessel wall, usually between the layer of ECs and the IEL, leads to the formation of soft fatty streaks also known as plaque. These streaks, which often contain cells of macrophage, SMC, and fibroblast lineage, may build over the course of a lifetime.

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

In many patients, the lesions undergo calcification to produce very hard lesions that require different therapeutic strategies than soft lesions. In either case, rather than cause the vessel to expand outward, these sustained, gradual processes encroach into the lumen of the vessel. Eventually luminal diameter becomes reduced to such a significant extent that blood flow is compromised. In the case of the coronary vasculature, inadequate blood flow leads to inadequate oxygen delivery to the heart which, as a muscle, cramps. This process is more commonly known as a heart attack or myocardial infarction (MI) and is usually the first symptom a patient exhibits that indicates vascular occlusive disease.

Vascular Response to Treatments - The treatments for vascular proliferative diseases are numerous and diverse, including percutaneous transluminal coronary angioplasty (PTCA or balloon angioplasty), bypass or interpositional grafting, carotid endarterectomy, hemodialysis vascular access grafts, transplant, and stents. All of these procedures lead to successful revascularization in the majority of cases. PTCA will be discussed to exemplify the events that occur immediately as a direct result of treatment (5-8). Endothelial denudation occurs, thus removing the barrier between the blood and the underlying components of the vessel wall. Typically, inflation of the balloon catheter also causes tearing, termed dissection or fracture, of the IEL and disruption of the media. Balloon inflation also causes plaque rupture and stretching of medial myocytes and SMCs, inducing proliferation of these cells. Because of the elastin in the vessel wall another response to stretching is elastic recoil, a process wherein the vessel wall attempts to return to the previous state. These processes lead to a long term compromise in the beneficial effects of ballooning. While larger acute gain in minimal lumen diameter (MLD) results in larger late loss in MLD and potentially higher restenosis rates, it is still best to achieve the maximum gain at the time of initial intervention (9).

These short term responses initiate a cascade of subsequent events. Thrombin and fibrin generation lead to platelet adhesion through the glycoprotein IIb/IIIa receptors to thrombogenic surfaces created by removal of ECs. Activated platelets release numerous factors including the vasoconstrictors thromboxane and serotonin. Thrombosis occurs along with the release of additional factors such as basic fibroblast growth factor (bFGF) and angiotensin, mitogens that promote SMC growth. The inflammatory response includes infiltration of macrophages, monocytes and neutrophils. Some signals cause medial SMCs and/or myocytes to undergo transformation, or dedifferentiation, from the normal contractile phenotype to a synthetic phenotype. These cells share characteristics of both SMCs and fibroblasts and have been called myo-fibroblasts. More recent reports suggest the possibility that adventitial fibroblasts undergo a similar dedifferentiation process (10).

Cells in the wall, predominantly of SMC origin, leave the quiescent stage of the cell cycle and begin to proliferate. In response to chemotactic factors like platelet-derived growth factor (PDGF) these cells then migrate to the luminal surface. Proliferation continues and is accompanied by elaboration of an extensive matrix that is fundamentally different from that in either the media or the adventitia. Re-endothelialization, or covering of the luminal surface with ECs, occurs to a variable extent and is hypothesized to limit the extent of luminal occlusion. The progressive accumulation of cells and matrix can lead to luminal narrowing and reduction of blood flow.

The early results of many interventional procedures are often remarkably good. Establishment of adequate flow relieves patient symptoms and requires little recovery time. The immediate events after intervention are important, normal processes that help maintain the structural and functional integrity of the vessel wall. These are

often considered a wound healing response to the injury, or arterial remodeling, induced by the intervention (11). In many patients, however, initiation of this cascade of physiological processes described above becomes excessive and leads to renarrowing, or restenosis, of the lumen and the return of symptoms. In this manner, intervention for the treatment of primary atherosclerosis causes the clinical indication of restenosis. However, these two diseases have very different etiologies, underlying biological mechanisms, and time of development.

There are no currently acceptable predictors of restenosis primarily because the clinical correlates are multifactorial (12-14). Patient factors include male gender, unstable angina, diabetes, cigarette smoking, and to a lesser extent congenital history, hypertension, and abnormal lipids. Lesion factors include number of vessels and lesions, location in the vasculature, length, and degree of calcification. Procedure factors include high incidence of restenosis, degree of dissection of the IEL, smoothness of the remaining lesion, and the establishment of good flow. Since patients surviving an initial MI receive interventional reopening treatment, they are typically monitored regularly at a fairly high cost. Thus, the positive perspective for patient care is that restenosis is predominantly an economic problem rather than a medical problem due to cost of monitoring and repeat procedures.

CLINICAL PROCEDURES

Balloon Angioplasty - PTCA, most commonly used for interventional treatment of both primary atherosclerosis and restenosis in the coronary vasculature of more than 400,000 patients per year in the United States, is limited by a variety of conditions (15). These include anatomic constraints such as often occur in total or nearly total occlusion, obstruction by intraluminal thrombus, dissections, hard calcified lesions, and diffuse disease. Rates of restenosis are 25-50% within 6 months. Quite often patients will have several angioplasties for recurrent restenosis, as many as 8 or more. Eventually the vessels are too diseased, making these patients candidates for grafts. Improvements in guidewires, balloon catheters, and guiding technologies have led to the use of PTCA for other vascular occlusive diseases, but the limitations of the method have led to development of many alternative devices.

Atherectomy - These devices physically remove tissue from the vessel wall. Directional coronary atherectomy (DCA) utilizes a high-speed cutter to remove lesions (16). In contrast to balloon angioplasty, DCA rarely causes dissection of the IEL. A frequent problem with atherectomy involves difficulty controlling the depth of excision which often results in cutting into, and removal of, medial SMCs. This action exacerbates the restenosis process. Nonetheless, DCA remains useful in the case of complex lesion morphologies, bifurcation lesions, and calcified plaques. It is increasingly common to use DCA followed by PTCA.

Rotational atherectomy, also known as the rotablator, is a very high speed burr that ablates and pulverizes inelastic, calcified lesion while leaving soft tissue relatively unaffected (17). This is also often followed by PTCA and is most useful for ostial lesions, lesions in tortuous vessels, and lesions in small diameter vessels (18).

Additional debulking atherectomy devices include the pullback catheter and ultrasound ablation catheter. In addition, the transluminal extraction atherectomy catheter is a cutting and aspiration device that works by plaque fissuring and atheroma extraction (15). Complex lesions in degenerated vein graft segments are

the most common application of this device. Finally, the use of fiberoptics to deliver laser light which causes thermal damage to the vessel wall is predominantly an adjunct to PTCA or for diffuse or calcified lesions (19).

Grafts - Vascular grafts are also important alternatives to angioplasty when lesions are complex or vessels are tortuous (20-22). In coronary arteries, the donor vessel is determined by the nature of the occlusion. While the mammary artery exhibits somewhat lower rates of restenosis than the saphenous vein, long vessels and multi-vessel lesions require use of the latter. These grafts are usually quite successful at reestablishing flow and typically have restenosis rates of less than 10% per year, developing over much longer time periods compared to angioplasty patients. However, the surgical procedures attendant to open heart surgery for coronary bypass grafts are much more difficult on patients, raising the incidence of morbidity and mortality while requiring much longer recovery times. Risk is predominantly influenced by the vessel grafted, the artery diameter, and the antithrombotic regimen used. In small diameter peripheral vessels, synthetic grafts made of polytetrafluoroethylene (PTFE) are common, particularly below the knee (23).

Endarterectomy - Carotid endarterectomy (CEA) is used to revascularize carotid arteries with reduced or no flow to prevent strokes or transient ischemic attacks in the brain (24). CEA is performed by shunting blood around plaques or restenotic lesions, opening the vessel longitudinally, and physically removing the diseased tissue. The vessels are then closed with either saphenous vein patch angioplasty or PTFE patches (25). These procedures have associated restenosis rates of 9-36%.

Hemodialysis Access Grafts - Patients requiring repetitive hemodialysis, predominantly diabetics, have forearm PTFE loop grafts that allow the for thrice weekly needs for vessel puncture without destroying native vessels (26-29). Since a limited number of vascular access sites are available, it is crucial to maintain the patency of these grafts. Occlusive disease occurs in about 37% of patients within 6 months. Balloon angioplasty is commonly used to reopen these diseased sites. However, restenosis rates rise with repetitive intervention. Graft failure eventually occurs and a new dialysis access graft is necessary.

Transplant Atherosclerosis - Despite improvements in the initial success of organ transplantation, long-term allograft survival remains poor (30). The major cause of death in these patients is cardiac allograft vasculopathy (CAV). This is an accelerated form of atherosclerosis characterized by substantial and rapid SMC proliferation that leads to luminal occlusion in nearly 45% of patients by 3 years post-transplantation. In contrast to the more common coronary artery disease, CAV is diffuse throughout the transplanted organ and therefore much more difficult to treat from an interventional strategy. One potential therapeutic paradigm could be to treat the donor organ ex vivo with anti-proliferative drugs.

Stents - Stents are used to treat many vascular proliferative diseases, in both the vessels of the coronary (31-33) and the periphery (34). These reduce the effects of elastic recoil in response to overstretch initiated by either stent deployment or balloon angioplasty. The resulting smooth luminal border is thought to play a large part in the success of stents but this is counterbalanced by their increased thrombogenicity. Intracoronary stent patients experience restenosis rates of about 29% at six months post-deployment.

DETERMINANTS OF THERAPEUTIC STRATEGIES

Clinical Definition of Restenosis - One of the key issues in the understanding of restenosis relates to the multiplicity of definitions used by clinicians and scientists (14, 35). Two fundamental differences have arisen based on analyses derived from either clinical or imaging endpoints. Clinical endpoints are often the first warning of compromised vascular perfusion for patients. In the case of limited coronary flow these include chest pains, MI, and death. If time allows, patients with the former two symptoms usually undergo exercise treadmill tests to evaluate cardiac function by examination of electrocardiograms. Imaging by angiography allows evaluation of vessel patency . In more sophisticated centers, quantitative angiography results in an actual value for the percent of vessel lumen open for flow (36). This value would be percent stenosis for primary atherosclerosis or percent restenosis for patients presenting after being treated for primary disease. Angiographers typically consider reduction of vessel luminal area by more than 50% to constitute restenosis. This cut-off is commonly used in clinical trials at follow-up six months after the initial intervention. However, clinical endpoints (i.e., patient symptoms) usually only occur when occlusion of flow is in the 70-95% range. Improvements in imaging such as intravascular ultrasound and magnetic resonance imaging (14) shelled help increase understanding and diagnosis of restenosis. However, this discord between endpoints has led to problems in the design, execution and evaluation of therapies (37).

Timing - A large number of drug, device, and drug-device combination therapies have been tested for prevention and treatment of restenosis. Because of the heterogeneous manifestation of restenosis, timing of therapeutic intervention is both crucial and complicated. While some drugs require chronic administration for effect, some forms of the disease require acute treatment. This dichotomy has led to tremendous diversity of strategies, often making comparative analyses difficult or impossible. Likewise, the nature of the diseased vessel can be a determinant: substantial or total occlusion of the coronary vasculature obviously requires acute intervention while peripheral veins tend to be more chronic in the expression of disease. These factors influence drug treatment duration and frequency. Finally, the target of the drug impacts greatly on the timing of administration relative to the interventional procedure. Drugs targeted at events initiated at the time of intervention, such as vasospasm, need to be delivered at or preceding intervention. Events occurring as a response to injury, such as SMC migration, are probably best treated by delivery after intervention and possibly for longer times.

Delivery - Along with drug discovery and development, drug delivery has become an area of intense focus. With the advent of catheters to reach specific locations in the vasculature, the idea of delivering drugs through these catheters has followed (38, 39). Local delivery clearly confers certain advantages: treatment immediately at the site, reduced amounts and cost of drug, lower systemic levels with consequent diminution of toxicological profiles, tolerance of formulations not necessarily suitable for systemic use, and reduced demand on drug bioavailability. Local delivery catheters have been designed employing a variety of strategies including a) active vs passive delivery, b) perfusion capabilities to allow continued blood flow during sustained delivery to the walls, c) isolation of specific segments of a vessel. The CorTrak (40) catheter uses iontophoretic current to rapidly drive charged drugs out of a balloon directly into the vessel wall. The InfusaSleeve (41), Microporous Infusion (42), and Dispatch (43) catheters have perfusion capabilities with pressure-driven

delivery. The double balloon catheter delivers to an isolated segment of the wall between two linear balloons. Finally, in the case of procedures where diseased vessels are accessible from the outside as in coronary artery bypass grafts, vessels can be directly treated or soaked in therapeutic agents ex vivo by a means analogous to that suggested for organ transplantation.

STATUS OF CLINICAL TRIALS

Many drugs have been tested for the prevention of restenosis and have been recently reviewed (44-46). While often providing protection in animal models, nearly all of these drugs have fallen short of acceptability in humans. Thus, a remaining difficulty in drug development is the lack of similarity between restenosis in man and other animals.

Small Molecules - To date, traditional small molecule drugs acting via a variety of mechanisms to prevent restenosis have been ineffective, inconsistent, or inadequate. Since angiotensin is a potent mitogen for SMC proliferation, inhibition of the synthetic enzyme, angiotensin-converting enzyme, was a reasonable target. Cilazapril proved to be effective in vitro and in animal models of restenosis but showed no significant differences from placebo regarding either clinical outcome or angiographically defined restenosis (47). Calcium channels are thought to mediate the vasospasm following balloon overstretch. Inhibitors of these channels such as diltiazem and veraprimil showed no significant improvement (48). Beta-adrenergic receptor antagonists which inhibited vasospasm in animal models were also ineffective in humans (49). Carvedial, a cardiovascular drug with multiple actions was very potent in animal models (50). Antiplatelet drugs such as aspirin, dipyridamole, and ticlopidine have been inadequate in many regimens (51). Trapidil, a platelet-derived growth factor antagonist, inhibited angiographic restenosis but had no effect on clinical events (52). The serotonin receptor antagonist, ketanserin, was also ineffective (53). Trials using the lipid-lowering drug lovastatin, the insulin growth factor1 inhibitor angiopeptin, or inhibitors of thromboxane A2 synthetase or thromboxane receptors were negative (6, 46). Molsidime, the nitric oxide snythetase inhibitor, was negative (54)

Natural Products - The antimitotic and antiproliferative agent colchicine was without effect in human trials (55). The thrombolytics tissue plasminogen activator (56) and urokinase (57) were unsuccessful. Prostacyclin, the most powerful antiaggregating substance known, showed no decrease in angiographic or clinical endpoints (58). Antiinflammatory agents such as dexamethasone and prednisolone were ineffective (6, 42). Potentially positive agents have been reported, including the omega fish oils, which led to a significant decrease in a combination of angiographic and/or clinical endpoints (59, 60) but is not widely used due to adverse side effects. The anticoagulant hirudin was recently reported to reduce early events but had no effect on long-term restenosis (61). The related compound hirulog was also without benefit (62). Fractionated low molecular weight heparin did not prevent restenosis in human trials (63).

Antibodies - The only approved agent for inhibition of clinical restenosis is Centocor's 7e3 (ReoPro), an antibody that antagonizes the action of the glycoprotein IIb/IIIa receptor to mediate platelet aggregation (64). This therapy is limited by the moderate, but significant reduction in clinical endpoints (24% restenosis at 6 months) and the necessity for intravenous delivery.

Stents - The use of stents to physically maintain the vessel wall in an expanded state has been compared to balloon angioplasty (15, 31-34). The positive attributes for stenting are prevention of dissections or flaps, improved results in vessels with complex lesions, and reduction of restenosis by prevention of recoil. The Palmaz-Schatz stent significantly reduced restenosis rates in clinical trials and is currently the only stent approved for use in man in the USA (65). Stents are also often used as a "bailout" procedure when angioplasty patients experience abrupt reclosure (66).

FUTURE DIRECTIONS

Critical determinants for successful identification of inhibitors of restenosis are study design, imaging, and predicative ability. Many studies have suffered from inadequate statistical power necessary to identify relatively small decreases in the 25-50% rates of restenosis. This can be overcome by increasing patient enrollment but the costs can become prohibitive. Better assessment of the need for pretreatment, the number and duration of doses, and actual measures of the effect or efficacy of an agent on the target are also required. More careful attention to the identification of clinical endpoints of restenosis will increase the comparative power of various treatments and trials but, again, also increase costs. Hopefully, improvements in vascular imaging will eventually allow the assessment of predictive and/or correlational variables with sufficient power to equal clinical endpoints.

Drug discovery and development will continue at a rapid pace (67). Improved and more specific antiplatelet agents and anticoagulants should be available soon. Inhibitors of growth factors (68), adhesion molecules (69), and their signal transduction pathways are increasingly specific and potent in inhibiting restenosis in animal models. Anti-oxidants such as probucol have also proven extremely effective in models (70). Selective targeting of toxic substances has been effective for basic FGF-toxin conjugates (71, 72).

The ability to deliver drugs is also evolving fast. Porous and microporous (73), channel (74), and hydrogel coated (75) balloons offer novel strategies. Coating stents with antithrombotics, antiproliferative agents, or cells such as ECs are in advanced stages of development (39). Photodynamic therapy using photosensitive nontoxic substances that are activated with light locally delivered through a catheter has inhibited restenosis in large animal models (76). Entrapment of drugs in biodegradable polymers, utilized in other diseases, may also be effective in restenosis (77). Local delivery of carefully titrated doses of low dose radiation to arrest cell division have almost completely abolished restenosis in large animal models (78).

Antisense oligonucleotides (ASOs) do not target the action of a protein as in the case with most traditional drugs. ASOs target production of the protein by inhibiting translation of the mRNA. This strategy has significantly inhibited restenosis in several animal models with ASOs targeted to genes involved in SMC proliferation such as the proto-oncogenes c-myc and c-myb, in addition to cdc2 kinase and proliferating cell nuclear antigen (38, 39, 46). In some cases, efficacy has been optimized using formulations of liposomes and viruses (79) or with porous infusion catheters (80). Additional potential targets for ASO intervention include the matrix molecules tenascin and osteopontin, and the fibroblast growth factor receptor1 (81).

Gene therapy holds great promise for prevention and treatment of restenosis (38, 39, 67). Infection of ballooned pig ileofemoral arteries (82) with locally delivered adenoviral vectors containing the thymidine kinase gene led to expression of the enzyme in the vessel wall. In response to gancyclovir, transduced cells were specifically killed. Similarly, infection with a constitutively active form of the retinoblastoma gene product inhibited neointimal formation in both rat carotid and pig femoral arteries (83).

Conclusions - Reduction in vascular proliferative disease such as restenosis is a very complex challenge related to the heterogeneous expression of this disease. While sharing the common features of luminal occlusion, typically a result of SMC proliferation and matrix deposition that encroach on the lumen, restenotic diseases are initiated by a wide variety of stimuli. Further, many of these stimuli occur by redundant but distinct processes. This bring into focus a crucial question: Is there a "magic bullet" strategy directed at a single target that is sufficiently and singularly critical for production of restenosis? Since the important, meaningful, and difficult answer to this question can only be appropriately obtained by studies in vascular proliferative disease in humans, it may not be imminently forthcoming. However, the advances discussed above should aid in the clarification, understanding and resolution of these issues.

References

1. J. Rhodin in "Handbook of Physiology: the Cardiovascular System and Vascular Smooth Muscle," Vol II, D. Bohr, A. Somlyo and H. Sparks, Eds., Am. Physiol. Soc., Bethesda, 1980, p. 1- 31.
2. K. Gould, Circulation, 90, 1558 (1994).
3. R. Ross, Nature,362, 801, (1993).
4. V. Dzau, G. Gibbons, J. Cooke and N. Omoigui, Circulation, 87, 705, (1993).
5. P. Lee, G. Gibbons and V. Dzau, Coronary Artery Disease, 4, 254 (1993).
6. H. Anderson, Disease-a-Month, 39, 613, (1993).
7. R. Lange, J. Willarad and L. Hillis, Am. J. Med. Sci., 306, 265 (1993).
8. E. O'Brian and S. Schwartz, Trends Cardiovasc. Med., 4, 169 (1994).
9. K. Beatt, P. Serruys, H. Juijten, B. Rensing, H. Suryapranata, P. deFeyter, M. van dan Brand, G. Laarman, J. Roelandt and G. van Es, J. Am. Coll. Cardiol., 19, 258 (1992).
10. N. Scott, C. Ross, R. Subramanian, F. Martin, L. Simonet and J. Wilcox, Circulation, 90, Supp. No 4-2, I-392, Abst. 2106 (1994).
11. J. Currier and D. Faxon, J. Am. Coll. Cardiol., 25, 516 (1995).
12. B. Rensing, W. Hermans, J. Vos, J. Tijssen, W. Rutch, N. Danchin, G. Heyndrickx, E. Mast, W. Wijns and P. Serruys, Circulation, 88, 975 (1993).
13. W. Hermans, B. Rensing, D. Foley, J. Tijssen, W. Rutsch, H. Emanuelsson, N. Danchin, W. Wijns, F. Chappuis and P. Serruys, J. Cardiovasc. Pharmacol., 22, Supp. No 4, S45, (1993).
14. D. Holmes and R. Schwartz, Coronary Artery Disease, 4, 229, (1993).
15. C. Wong, M. Leon and J. Popma, Coronary Artery Disease, 4, 243, (1993).
16. V. Umans, A. Robert, D. Foley, W. Wijns, E. Haine, P. deFeyter and P. Seurrys, J. Am. Coll. Cardiol., 23, 49 (1994).
17. S. Ellis, J. Popma, M. Buchbinder, I. Franco, M. Leon, K. Kent, A. Pichard, L. Statler, E. Topol and P. Whitlow, Circulation, 89, 882 (1994).
18. U. Dietz, R. Erbel, H.J. Rupprecht, S. Weidmann and J. Meyer, Br. Heart J., 70, 327 (1993).
19. F. Litvack, N. Eigler, J. Margolis, D. Rothbaum, J. Bresnahan, D. Holmes, W. Untereker, M. Leon, K. Kent, A. Pichard, S. King, Z. Ghazzal, F. Cummins, D. Krauthamer, I. Palacios, P. Block, G. hartzler, W. O'Neill, M. Cowley, G. Roubin, L. Klein, P. Frankel, C. Adams, T. Goldenberg, J. Laudenslager, W. Grundfest and J. Forrester, J. Am. Coll. Cardiol., 23, 323 (1994).
20. S. King, N. Lembo, W. Weintraub, A. Kosinski, H. Barnhart, M. Kutner, N. A.azrake, R. Guyton and X.-Q. Zhao, New England J. Med., 331, 1044 (1994).
21. M. Paz, J. Lupon, X. Bosch, J. Pomar and G. Sanz, Ann. Throac. Surg., 56, 1101 (1993).
22. S. Nikkari and A. Clowes, Ann. Med., 26, 95 (1994).
23. F. Veith, S. Gupta, E. Ascer, S. White-Flores, R. Samson, I. Scher, J. Towne, V. Bernhard, P. Bonier, W. Flinn, P. Astelford, J. Yao and J. Bergan, J. Vasc. Surg., 3, 104 (1986).
24. D. Healy, A. Clowes, R. Zierler, S. Nicholls, R. Berglelin, J. Primozich and D. Strandness, Stroke, 20, 1138 (1989).

25. D. Katz, S. Snyder, R. Gandhi, J. Wheeler, R. Gregory, R. Gayle and F. Parent, J. Vasc. Surg., 19, 198 (1994).
26. A. Rinehart, C. Herzog, A. Collins, J. Flack, J. Ma and J. Opsahl, Am. J. Kidney Dis., 25, 281 (1995).
27. C. Schwartz, C. McBrayer, J. Sloan, P. Meneses and W. Ennis, Radiol., 194, 337 (1995).
28. R. Kanterman, T. Vesely, T. Pilgram, B. Guy, D. Windus and D. Picus, Radiol., 195, 135 (1995).
29. D. Worwerk, R. Guenther, H. Mann, K. Bohndorf, P. Keulers, G. Alzen, M. Sohn and D. Kistler, Radiol., 195, 140 (1995).
30. J. Hosenpud, G. Shipley and C. Wagner, J. Heart Lung Transplant, 11, 9 (1992).
31. C. Chan, A. Tan, T. Koh, P. Koh, K. Lau, S. Gunawan, B. Chakraborty and A. Ng, Am. J. Cardiol., 75, 23 (1995).
32. Y. Rozenman, C. Lotan, M. Mosseri and M. Gotsman, Am. J. Cardiol., 75, 84 (1995).
33. A. Rodriguez, O. Santaera, M. Larribau, M. Fernandez, R. Sarmiento, N. Balino, J. Newell, G. Roubin and I. Palacios, Circulation, 91, 1397 (1995).
34. A. Long, M. Sapoval, B. Beyssen, M. Auguste, Y. LeBras, A. Raynaud, G. Chatellier and J. Gaux, Radiol., 194, 739 (1995).
35. R. Kuntz and D. Baim, Circulation, 88, 1310 (1993).
36. P. Serruys, D. Foley, R. Kirkeeide and S. King, Am. Heart J., 126, 1243 (1993).
37. S. Nakamura, D. Mahon, B. Maheswaran, D. Gutfinger, A. Colombo and J. Tobis, J. Am. Coll. Cardiol., 3, 633 (1995).
38. R. Riessen and J. Isner, J. Am. Coll. Cardiol., 23, 1234 (1994).
39. A. Lincoff, E. Topol and S. Ellis, Circulation, 90, 2070 (1994).
40. A. Fernandez-Ortiz, B. Meyer, A. Mailhac, E. Faik, L. Badimon, J. Fallon, V. Fuster, J. Chesebro and J. Badimon, Circulation 89, 1520 (1994).
41. A. Kaplan, M. Vandormeal, A. Bartorelti, M. Hofmann, H. Stoerger, J. Simpson and N. Reifart, J. Am. Coll. Cardiol., 286A Abst 772-3 (1995).
42. D. Eccleston, M. Horrigan and S. Ellis, Interven. Cardiol. Monitor, 1, 33 (1995).
43. E. Santoian, N. Tarazona, M. Taylor, R. Kovach, S. Malosky and C. Dennis, J. Am. Coll. Cardiol., 302A, Abst. 984-25.
44. J.-P. Herrman, W. Hermans, J. Vos and P. Serruys, Drugs, 46, 18 (1993).
45. S. Franklin and D. Faxon, Coronary Artery Disease, 4, 232 (1993).
46. L. Feldman, R. Riessen and P. Steg, Fundam. Clin. Pharmacol., 9, 8 (1995).
47. MERCATOR Study Group, Circulation, 86, 100 (1992).
48. E. Hoberg,, R. Dietz and U. Frees, Br. Heart J., 71, 254 (1994).
49. S. Johansson, C. Lamm, G. Bondjers, H. Emanuelsson and A. Hjalmarson, Am. J. Cardiol., 66, 915 (1990).
50. E. Ohlstein, S. Douglas, C. Sung, T.-L. yue, C. Louden, A. Arleth, G. Poste, R. Ruffolo and G. Feuerstein, Proc. Natl. Acad. Sci., USA, 90, 6189 (1993).
51. J. van der Meer, H. Hillege, G. Kootstra, C. Ascoop, M. Pfisterer, W. vanGilst and K. Lie, Lancet, 342, 257 (1993).
52. A. Maresta, M. Balducelli, L. Cantini, A. Casari, R. Chioin, M. Fabbri, A. Fontanelli, P. Preti, S. Repetto, S. deServi and E. Varani, Circulation, 90, 2710 (1994).
53. P. Serruys, W. Klein, J. Tijssen, W. Rutsch, G. Heyndrickx, H. Emanuelsson, S. Ball, O. Decoster, E. Schroeder, H. Liberman, E. Eichhorn, J. Willerson, H. Anderson, F. Khaja, R. Alexander, D. Baim, R. Melkert, J. vanOene and R. vanGool, Circulation 88, 1588 (1993).
54. H. Emanuelsson, J. Bagger, R. Balcon, K. Beatt, M. Foegh, J. Piessens and J. Popma, J. Am. Coll. Cardiol., 23, Abst. 59A (1994).
55. J. O'Keefe, B. McCallister, T. Bateman, D. Kuhnlein, R. Ligon and G. Hartzler, J. Am. Coll. Cardiol., 19, 1597 (1992).
56. P. Gurbel, F. Navetta, B. Muhlstein, A. Tenaglia, D. Muller, E. Bates, C. Davidson, M. Miller, F. Aquirre, J. Hermiller, C. Tommaso, L. Berdan, Pl Owens, G. Beauman, J. Leimberber, E. Bovill and E. Ohman, J. Am. Coll. Cardiol., 347A, Abst. 1001-25 (1995).
57. J. Hartmann, L. McKeever, W. O'Neill, C. White, P. Whitlow and E. Enger, J. Am. Coll. Cardiol., 149A, Abst 738-6 (1995).
58. A. Gershlick, D. Spriggins, S. Davies, Y. Court, J. Timmins, A. Timmins, M. Rothman, C. Layton and R. Balcon, Br. Heart J., 71, 7, (1994).
59. I. Bairati, L. Roy and F. Meyer, Circulation, 85, 950 (1992).
60. J. Gapinski, J. vanRuiswyk, G. Heudebert and G. Schectman, Arch Intern. Med., 153, 1595 (1993).
61. P. Serruys, J. Deckers and P. Close, Circulation, 90, I-394, Abst. 2115 (1994).
62. C. Meckel, W. Ahmed, J. Ferguson, J. Strony, D. Marks, D. Manuelian, B. Adelman and J. Bittl, Circulation, 90, I-64, Abst. 336, (1994).
63. D. Faxon, T. Sprio, S. Minor, G. Cote, J. Douglas, R. Gottlieb, R. Califf, K. Dorosti, E. Topol, J. Gordon and M. Ohmen, Circulation, 90, 908 (1994).
64. J. Lefkovits, K. Anderson, H. Weisman and E. Topol, New Engl. J. Med., 330, 956, (1994).
65. A. MacIsaac, J. Elliott, S. Ellis, E. Tuzcu, I. Franco, R. Raymond and P. Whitlow, Circulation, 90, I -611, Abst. 3295 (1994).

66. P. Aubry, T. royer, C. Spaulding, B. Lancelin, R. Faivre, M. Henry, B. Livarek, P. Rioux, B. Valette, C. Masquet, M.-C. Morice and G. Zemour, Circulation 90, I-124, Abst. 664 (1994).
67. S. Epstein, E. Speir, E. Unger, R. Guzman and T. Finkel, J. Am. Coll. Cardiol., 23, 1278 (1994).
68. A. Doherty and J. Keiser, Ann. Reports in Med. Chem., 28, 89 (1993).
69. Y. Jang, A. Lincoff, E. Plow and E. Topol, J. Am. Coll. Cardiol., 24, 1591 (1994).
70. J. Schneider, B. Berk, M. Gravanis, E. Santoian, G. Cipolla, N. Tarazona, B. Lassegue and S. King, Circulation, 88, 628 (1993).
71. S. Biro, C. Siegall, Y. Fu, E. Speir, I Pastan and S. Epstein, Circ. Res., 71, 640 (1992).
72. W. Casscells, D. Lappi and B. Olwin, Proc. Natl. Acad. Sci. USA, 89, 7159 (1992).
73. C. Lambert, J. Leone and S. Rowland, Coronary Artery Dis., 4, 469 (1993).
74. M. Hong, S. Wong, A. Farb, M. Mehlman, R. Virmani, J. Barry and M. Leon, Coronary Artery Dis., 4, 1023 (1993).
75. M. Azrin, J. Mitchel, D. Fram, C. Pedersen, R. Cartun, J. Barry, L. Bow, D. Walters and R. McKay, Criculation 90, 433 (1994).
76. M. Moran, K. March, R. Wilendky, K. Mehki, D. Hathaway, Circulation, 90, I-143, Abst. 762 (1994).
77. I. Tai and A. Sun, FASEB J, 7, 1061 (1993).
78. R. Waksman, K. Robinson, I. Crocker, M. Gravanis, G. Cipolla and S. King, Circulation, 91, 1533 (1995).
79. R. Morishita, G. Gibbons, K. Ellison, M. Nakajima, H. von der Leyuen, L. Zhang, Y. Kaneda, T. Oghara and V. Dzau, J. Clin. Invest., 93, 1458 (1994).
80. Y. Shi, A. Fard, A. Galeo, h. Wutchinson, P. Vermani, G. Dodge, D. Hall, F. Shaheen and A. Zalewski, Circulation, 90, 944 (1994).
81. L. Denner, R. Dixon, S. Eskin, H. McAllister, A. Rege, D. Stacy, L. Sturgis, N. Kint, H. Tian, C. Munsch, C. Parnis, P. Capek and J. Willerson, Circulation 90, I-296, Abst. 1592 (1994).
82. T. Ohno, D. Gordon, H. San, V. Pompili, M. Imperiale, G. Nabel and E. Nabel, Science, 265, 781, (1994).
83. M. Chang, E. Barr, J. Seltzer, Y.-Q. Jiang, G. Nabel, M. Parmacek and J. Leiden, Science, 267, 518 (1995).

Chapter 8. Anticoagulant Strategies Targeting Thrombin and Factor Xa

Robert M. Scarborough
Medicinal Chemistry Research
COR Therapeutics, Inc.
South San Francisco, California 94080

Introduction - The development of novel anticoagulant agents to replace or supplement existing therapeutic regimens for the treatment of thrombosis continues to receive significant and growing attention (1-3). The blood coagulation system involves the conversion of a variety of inactive enzymes (zymogens) into active enzymes and is the target of existing anticoagulant drugs. Thrombin, a serine protease with trypsin-like specificity, plays a pivotal role in the coagulation cascade through the final conversion of fibrinogen to fibrin. Thrombin is also the most potent platelet agonist known and therefore positions thrombin as a central mediator in both thrombosis and in normal hemostasis. The major classes of compounds that are currently used as anticoagulants all indirectly inhibit the activity of thrombin. Heparin, through its action on the plasma proteins, antithrombin III and heparin cofactor II, indirectly inhibits thrombin in addition to other proteases (4). Similarly, the coumarin class of oral anticoagulants inhibit the synthesis of vitamin K-dependent protease zymogens which occupy key points within the coagulation cascade (factors II,VII, IX,and X).

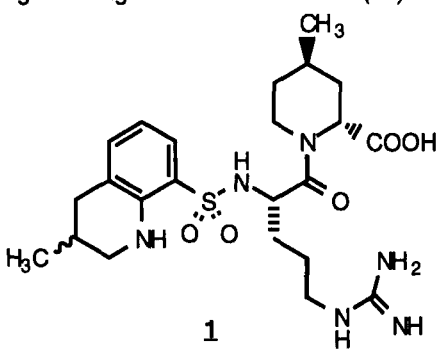
Side effects, limitations of efficacy and bleeding complications with these agents have stimulated the search for novel, more direct and specific inhibitors of key coagulation enzymes (5). In addition to summarizing the recent clinical experiences with direct thrombin inhibitors, this chapter will cover the development of recently described specific small molecule direct thrombin inhibitors. Finally, novel approaches to inhibit factor Xa, the enzyme within the prothrombinase complex ultimately responsible for the conversion of inactive prothrombin to the procoagulant thrombin, will be reviewed.

RECENT CLINICAL RESULTS WITH DIRECT THROMBIN INHIBITORS

Due to the the central role that thrombin plays in thrombosis, direct inhibitors of thrombin have been the early focus of efforts to develop new antithrombotic regimens (5,6). This focus derives from the clinical usage of heparin and to a certain degree the recent introduction of the low molecular weight heparins (LMWH) (4). Heparin has been widely used as an anticoagulant for a number of years in a variety of clinical settings of severe or life threatening thrombosis, including the treatment of venous thrombosis, pulmonary embolism, prevention of thrombosis post myocardial infarction, as adjunctive to thrombolytic therapy, during angioplasty procedures and in the treatment of patients with unstable angina (4). Although heparin has proven clinical benefits, limitations of its effectiveness, patient variability to its effects and certain side effects have suggested that direct thrombin inhibitors might be more ideal antithrombotic agents. Direct thrombin inhibitors would bypass the need for antithrombin III cofactor activity and would theoretically have more predictable anticoagulant action as well as improved potency over heparin through their abilities to inhibit both fluid-phase and clot-bound thrombin activity. These considerations have inspired the development of direct thrombin inhibitors as novel agents (7).

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Argatroban - The first small molecule direct thrombin inhibitor to be developed and studied in the clinic is argatroban (MD-805, 1); reported to have a K_i for thrombin inhibition of 19 nM (8). Argatroban is an active-site directed competitive inhibitor containing an arginyl-amide functionality that is recognized by thrombin's specificity pocket. The first clinical studies with this parenteral agent were conducted in the early 1980's. However, recent clinical evaluation of this agent has been conducted in a small group of patients with unstable angina pectoris. While demonstrating potent effects on clotting and thrombin activity, argatroban displayed both clinical and biochemical rebound phenomenon characterized by repeat ischemic events and unabated thrombin generation during infusions and significantly elevated thrombin and coagulation activity post drug infusion (9,10). One potential mechanism responsible for this phenomenon might be the negative effects of direct thrombin inhibitors on the thrombin-thrombomodulin complex, that mediates the conversion of protein C to activated protein C (APC), a potent feedback anticoagulant factor regulating the coagulation cascade. Another potential mechanism might be related to the relatively fast off-rate of argatroban which may afford incomplete thrombin inhibition during and post administration. Rebound phenomena with other direct thrombin inhibitors have not been observed in other clinical trials of direct thrombin inhibitors and general conclusions concerning these agents cannot be drawn (10).



1

Hirudin - The medicinal leech (*Hirudo medicinalis*) contains one of the most potent reversible inhibitors of thrombin isolated to date with a reported subnanomolar K_i value (11). This inhibitor was isolated more than 30 years ago by Markwardt and was named hirudin. Hirudin is a compact, disulfide linked peptide containing 65 amino acids that binds to thrombin in a bidentate manner not dissimilar to fibrinogen binding to thrombin. The carboxyl terminal tail of hirudin contains a large number of acidic residues, including a sulfated tyrosine residue, and binds to the anion-binding exosite used in substrate recognition by thrombin. The remainder of the hirudin molecule including the disulfide linked core region, and the amino terminus, binds to an apolar site on the surface of thrombin close to the enzyme active site but in a manner such that access by substrates is effectively blocked when hirudin is bound (12). Although there are additional contact sites of hirudin with thrombin, hirudin's unique mode of binding imparts unsurpassed specificity and potency as a thrombin inhibitor. The development of recombinant forms of desulfato-hirudin, (CPG 39393, HBW 023), has enabled extensive preclinical evaluation of hirudin in animal models of thrombosis, and was critical for supplying sufficient quantities for clinical trials (13).

Initial clinical experience in human volunteers using single or repeated subcutaneous injections has demonstrated that hirudin is well tolerated and exhibits dose-dependant effects on coagulation parameters (14). Pilot studies of recombinant hirudin have been subsequently studied in the treatment of deep vein thrombosis using

subcutaneous administration (15), versus heparin in patients with unstable angina undergoing coronary angioplasty (16), and in conjunction with tissue-type plasminogen activator (tPA) for the treatment of myocardial infarction in the Thrombolysis in Myocardial Infarction (TIMI) 5 trial (17) and with streptokinase in the TIMI 6 trial (18). In each of these settings, hirudin was an effective and predictable anticoagulant which did not display, even at the highest doses studied, any increase in major hemorrhage relative to heparin. In these trials, 437 patients received hirudin and while not powered to detect statistically significant differences in efficacy and safety between heparin and hirudin, the data revealed trends, with the hirudin-treated patients yielding greater patency rates in TIMI 5, and lower incidences of nonfatal reinfarction and death in hirudin-treated patients in TIMI 6. Many additional studies have been conducted with hirudin and these have been recently reviewed (2).

Three simultaneous investigations studying hirudin versus heparin in large scale phase III clinical trial settings have been initiated. One of these trials was initiated in acute coronary syndromes by The Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) IIa Investigators (19). The second was a trial of hirudin in acute myocardial infarction by the TIMI 9A investigators (20) and the third trial was referred to as the r-Hirudin for Improvement of Thrombolysis phase III study (HIT-III) (21). All three trials have been stopped earlier than planned. In the GUSTO IIa trial, recruitment of 12,000 patients was planned but was terminated following recruitment of the first 2,564 patients, due to an excess of intracranial hemorrhagic events in both heparin and hirudin groups. Similarly, the TIMI 9A trial which planned for the recruitment of ~3,000 patients was suspended after the enrollment of 757 patients, again due to excessive major spontaneous hemorrhage in heparin and hirudin-treated groups (20). The HIT-III trial was also a large Phase III trial originally planned to enroll 7,000 patients but was stopped after recruitment of only 302 patients (21). The HIT-III trial was different from the TIMI 9A and GUSTO IIa trials in that observations of increased rates of intracranial hemorrhage were only observed in the hirudin group in spite of the lower doses of hirudin (0.4 mg/kg body wt bolus and 0.15 mg/kg/hr) used versus the hirudin doses administered in the TIMI 9A and GUSTO IIa trials (0.6 mg/kg body wt bolus and 0.2 mg/kg/hr). However, both the TIMI 9A and GUSTO IIa trials used an approximately 20% higher dose of heparin than in the previous TIMI 5, TIMI 6 and GUSTO I investigations. This increased anticoagulation led to an apparent twofold risk of hemorrhagic stroke in patients receiving thrombolytic therapy. As a result of these clinical experiences it has been suggested that the therapeutic range of hirudin as an adjunct to thrombolysis may be smaller than previously thought, and that reappraisal of dose findings should be considered. Both the TIMI 9A and GUSTO IIb trials have been reinitiated with lower doses of hirudin (0.1 mg/kg body wt bolus and 0.1 mg/kg/hr infusion) and heparin (1000 U/hr non-weight adjusted). The completion and evaluation of these trials will determine whether a suitable therapeutic window exists for hirudin and possibly other direct thrombin inhibitors in the setting of thrombolysis (22). It should be noted that excessive bleeding has not been observed in other acute coronary settings such as in unstable angina with this agent where clinical efficacy has been demonstrated (2).

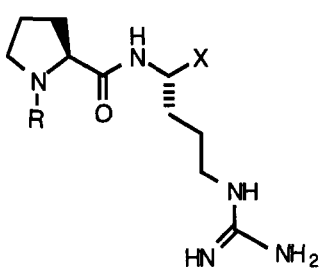
Hirulog - Hirulog, H-D-Phe-Pro-Arg-Pro-$(Gly)_4$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu Glu-Tyr-Leu-OH, BG-8967 (**2**) is a 20-residue synthetic peptide designed to mimic the potent, direct thrombin inhibitor hirudin (23). Hirulog interacts with thrombin in a bivalent manner, through its acidic rich tail to the anion-binding exosite of thrombin in a manner analogous to hirudin, and through the interaction of the amino-terminal D-Phe-Pro-Arg-Pro sequence to the active site of the enzyme. The polyglycine spacer of hirulog serves to position these two binding interactions (24). Hirulog inhibits thrombin

with a K_i = 2.3 nM, and has been studied extensively in animal models of arterial thrombosis and thrombolysis (25). The initial clinical evaluation of this agent has found that it is reasonably well tolerated and affords linear dose-dependant anticoagulant activity in the settings of unstable angina, myocardial infarction, and coronary angioplasty (26-28). Based on these pilot studies, a 4300 patient study of hirulog in coronary angioplasty was initiated and has been recently completed. Hirulog displayed a lower bleeding incidence compared to heparin but did not show a statistically significant benefit over heparin at fairly high doses (2.2 mg/kg bolus and 1.0 mg/kg/hr infusion) in this study (29). The future clinical development of hirulog is uncertain.

LOW MOLECULAR WEIGHT-DIRECT THROMBIN INHIBITORS

With the recent advent of greater detail in the understanding of the structure of thrombin and similar serine proteases through X-ray crystallographic determination of enzyme-inhibitor complexes, the ability to rationally design very potent and selective inhibitors of thrombin has flourished (30,31). The potential therapeutic advantages and chemistry of these novel direct thrombin inhibitors have been reviewed extensively (5-7). Thus, this section will focus on the more recent promising inhibitors that appear to be be in development phases prior to clinical evaluation.

Transition State Reversible Inhibitors - Thrombin inhibitors of this class rely on the substrate binding modes and the catalytic mechanism of thrombin to bind tightly to the enzyme and inhibit its catalytic activity. This binding mode is achieved in a fashion that is quite similar to the "transition state" intermediate that substrates assume as the peptide-amide carbonyl adopts a tetrahedral configuration. This is accomplished when the active site Ser-195 hydroxy group of thrombin adds to the carbonyl of the scissile bond. Most of the thrombin inhibitors of this class reported to date utilize an optimized tripeptide sequence with a polarizable functionality susceptible to attack by the active site serine of the enzyme (5). A model tripeptide, substrate-like binding sequence comes from the pioneering work on the chloromethyl ketone analog of the D-Phe-Pro-Arg sequence called PPACK **3** (32). Efegatran (LY294468, GYKI-14766, **4**), a slow-tight binding inhibitor of thrombin with a reported K_i = 7 nM, contains a similar tripeptide sequence with an aldehyde functionality. Efegatran has demonstrated potent effects in animal models of thrombosis and thrombolysis (33,34). Efegatran has been administered intravenously to a small group of unstable angina patients (35). In this trial, all dose levels of the drug were tolerated and displayed dose-dependent inhibition of coagulation parameters. Tripeptide arginals such as efegatran have been the subject of extensive structure-activity studies to optimize potency, oral bioavailability and selectivity versus other members of the serine protease family of coagulation and fibrinolytic enzymes (36). Other interesting members of this family are the N-Me-phenylglycine-containing inhibitor LY287045 **5**,and the (D)-1-Tiq-containing arginal **6**



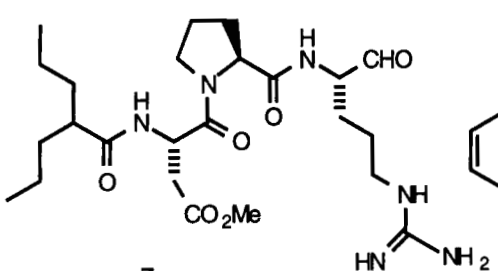
3 R = D-Phe; X = $COCH_2Cl$

4 R = D-MePhe; X = CHO

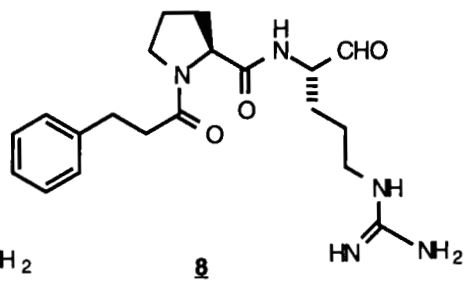
5 R = D-MePhg; X = CHO

6 R = D-1-Tiq; X = CHO

(1-Tiq = 1-tetrahydroisoquinoline), each displaying greatly enhanced inhibitory selectivity for thrombin versus plasmin or tissue plasminogen factor. Good selectivity is likely to be required if such inhibitors are to be used as adjunctive agents to thrombolytic therapy (36). Bioavailability in cynomolgus monkeys (36%) has been demonstrated for the tripeptide arginal CVS-1123 **7** with a K_i = 1.4 nM (37). Also, an acylated dipeptide arginal, BMY 44621 **8** has been reported with a K_i = 140 nM. Although this compound is less potent than other members of the arginal family, it has time-independent binding properties and prolongs clotting times following intravenous and oral administration and is effective in animal models of arterial and venous thrombosis (38).

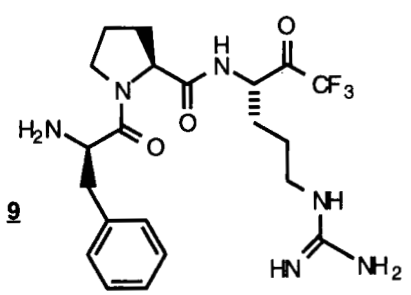


7

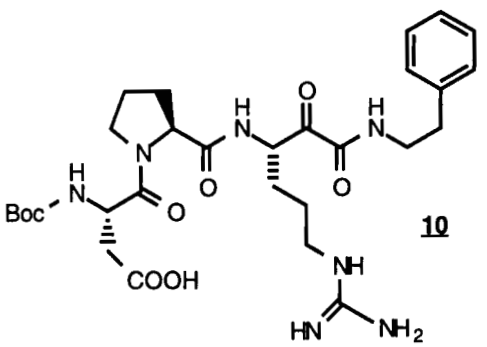


8

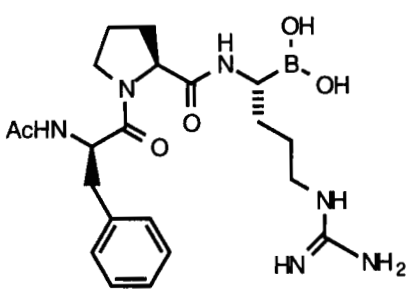
Examples of other polarizable functionalities which have been incorporated to bind at the active site are exemplified by the trifluoromethylketone-containing inhibitor MDL73756 **9** with a K_i < 1nM (39), the ketoamide **10** with a K_i = 11 nM (40), the peptide boronic acid DuP 714 **11** (41), and the peptide boronic acid esters



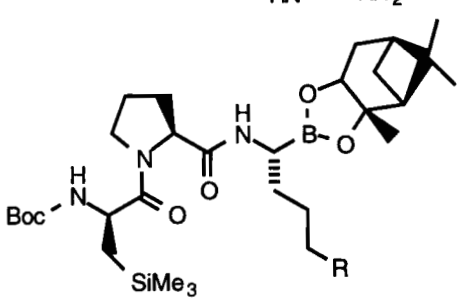
9



10



11



12 R = -NH-C(NH_2)=NH

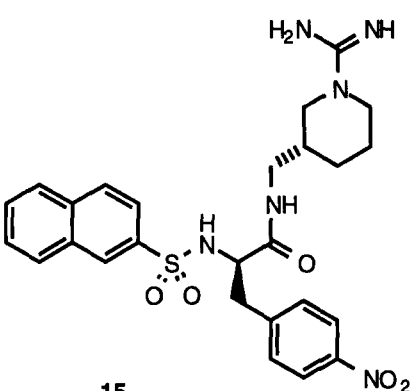
13 R = -OMe

SDZ217766 12, and SDZ219349 13. The latter inhibitor 13 contains a methoxypropyl sidechain substituted for the typical arginyl sidechain that imparts greater thrombin selectivity to this inhibitor over plasmin or trypsin (42,43). Another novel inhibitor, CVS-995 14 , with a K_i of 2-3 pM is a 19-amino acid containing peptidomimetic that utilizes the alpha-ketoamide functionality in combination with the multi-binding motifs, similar to hirulog, to inhibit thrombin (44).

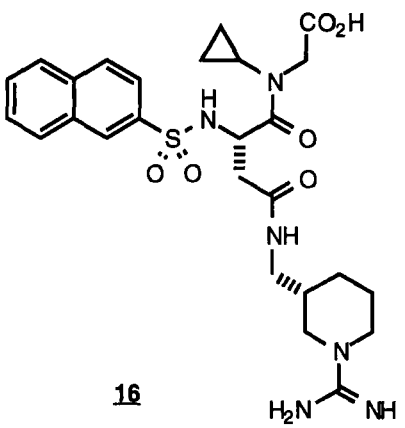
$(CH_3CH_2CH_2)_2$-CHCO-Asp-Pro-Arg ψ [COCO]-$(Gly)_5$-Asn-Gly-Asp-Phe-Glu-Glu-Ile-Pro-Glu-Tyr-Leu-OH

14

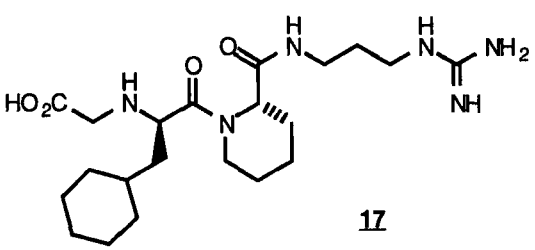
Active Site Inhibitors- Recently described thrombin inhibitors of this class utilize either "substrate binding modes" or "two distinct inhibitor binding modes" to achieve specific, competitive and reversible inhibition of thrombin (45). Two examples of novel (aminoiminomethyl)piperidine containing thrombin inhibitors are Ro 46-2548 15 (K_i = 10 nM) and Ro 46-6240 16 (K_i = 0.27 nM) (45). Although these inhibitors are structurally quite similar, two distinct "inhibitor binding modes" have been described for them and they also display distinct selectivity for inhibition of clot-bound thrombin compared with fluid-phase thrombin (46). The inhibitor 16, which is three times more potent in inhibiting clot-bound thrombin has also been studied in animal models of thrombosis and has been chosen as a clinical candidate (47). It is possible that inhibitors with greater activity versus clot-bound thrombin may be more potent in vivo and may display fewer bleeding complications. Another inhibitor of this class, Inogatran 17, has also progressed into Phase I clinical studies (48). Inogatran which has a K_i = 15 nM, is short acting ($T_{1/2}$ = 1 hr), is not metabolized, and is intended for intravenous use.



15



16



17

RECENT RESULTS WITH INHIBITORS OF FACTOR Xa/ PROTHROMBINASE

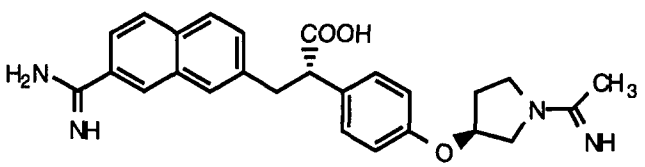
Recent clinical trial experience has clearly demonstrated that even in the face of high grade thrombin inhibition, there is unabated thrombin synthesis (49). At vascular lesion sites, locally generated thrombin may overcome systemic antithrombin therapy due to the localized amplification of thrombin synthesis via the prothrombinase complex (50,51). In light of these observations, inhibition of prothrombinase complex assembly or inhibition of the enzymatic activity of the prothrombinase complex may be attractive alternate targets for anticoagulant development. Mechanistically, the prothrombinase complex which converts prothrombin to active thrombin is situated at the convergence of the intrinsic and extrinsic coagulation pathways. The complex is formed when activated factor Xa and its cofactor Va are assembled on phospholipid surfaces, most notably activated platelets. The important role of the prothrombinase complex in sustaining ongoing thrombosis is beginning to emerge from studies of factor Xa/ prothrombinase inhibitors in animal models of thrombosis (52-55). In this chapter will be reviewed the discovery and development of these novel inhibitors of thrombin synthesis.

Inhibitors of Prothrombinase Complex Assembly- One of the first demonstrations that the prothrombinase complex was a potential target for developing novel anticoagulants was illustrated by the inhibition of the assembly of active prothrombinase complexes in vitro to afford an anticoagulant effect. The model for such an inhibitor was derived from the observation that binding of factor Xa to factor Va on membrane surfaces was independent of the active site of factor Xa (56). Thus, it was demonstrated that a covalently blocked, active site modified form of factor Xa (DEGR-Xa), could effectively increase clotting times in plasma and inhibit fibrinogen consumption in a baboon model of disseminated intravascular coagulation (DIC). This was achieved through the ability of DEGR-Xa to compete with factor Xa for incorporation into active prothrombinase complexes (57). These observations have been extended to other inhibited forms of factor Xa such as a recombinant form of factor Xa with a disabled catalytic triad called factor Xai (58). These prothrombinase complex assembly inhibitors have been shown to be effective anticoagulants in models of non-occlusive deep vein thrombosis (59) and in a canine arterial thrombosis model (54).

Inhibitors of Factor Xa in Active Prothrombinase Complexes- The study of thrombin structure and function has been greatly aided by the medicinal leech derived inhibitor, hirudin. Similarly, other hematophagous organisms have played an important role in studying the functional role of factor Xa in coagulation (59). Two extremely potent and selective inhibitors of factor Xa have been recently described (60,61). The Mexican leech (*haementeria officinalis*) yielded the selective and tight binding subnanomolar inhibitor of factor Xa called antistasin (ATS) which is a 119-amino acid, cysteine-rich, single chain polypeptide whose structure and function have been reviewed (59). A recombinant form of antistasin, r-ATS, has been studied in vivo in a variety of thrombosis models and has displayed potent antithrombotic activity (62). A second potent inhibitor of factor Xa has been isolated from the soft tick (*Ornithodoros moubata*) and designated tick anticoagulant peptide (TAP) (61). TAP is a 60-amino acid disulfide linked monomeric protein with limited homology to the Kunitz-type serine protease inhibitor family. The structure and function of TAP have also been extensively explored (63,64). Although TAP's mechanism of inhibition of factor Xa is very different from Kunitz-type serine protease inhibitors and it is still incompletely understood, TAP may inhibit the enzyme by covering over the active site of the enzyme in a fashion similar to the hirudin-thrombin complex (12). Recombinant TAP has been extensively studied in animal models of thrombosis (63). In one direct comparison of rTAP and

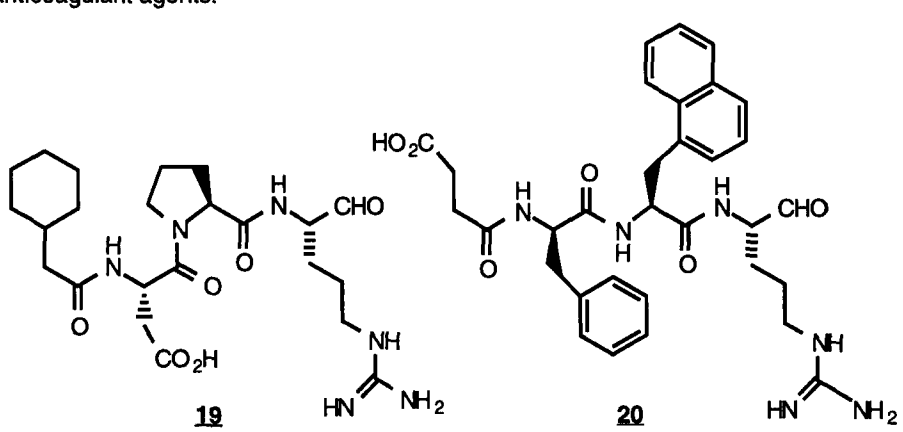
hirudin, rTAP more effectively maintained post-thrombolysis vessel patency in dogs, lending support to the hypothesis that ongoing thrombin generation, at least in the setting of thrombolysis is more effectively controlled with factor Xa inhibition than via thrombin inhibition (52).

Active Site Reversible Inhibitors- Until recently, efforts to prepare highly active, specific inhibitors of factor Xa or inhibitors of the enzymes in active prothrombinase complexes has been limited (65). The bisamidine mimic DX-9065a **18**, is the first potent, synthetic, and specific inhibitor of factor Xa to be reported (66). Although the structure of **18** is reminiscent of other potent but nonspecific inhibitors of the bis(amidinoaryl) class, this inhibitor was designed to be specific for factor Xa (K_I = 41 nM), with little effect on thrombin and other serine proteases except trypsin (67). Molecular modeling of this inhibitor with a factor Xa model derived from trypsin crystallographic coordinates, has suggested the molecular basis of the observed specificity (66). DX-9065a has been studied in a variety of venous and arterial thrombosis animal models and displays good antithrombotic activity when administered intravenously and poor to modest bioavailability when administered orally (68).



18

Transition State Reversible Inhibitors- Several preliminary reports of novel transition state, reversible inhibitors of factor Xa have appeared and are represented by the arginal compounds **19** and **20** which have IC_{50} values of 31 and 23 nM respectively (69,70). Arginal **19** is not specific for factor Xa inhibition versus inhibition of thrombin or plasmin. However, **20** is approximately 1000-fold more specific for factor Xa inhibition versus thrombin or t-PA. Improvement in the activity and specificity of novel factor Xa inhibitors appears to be a promising approach to the development of novel anticoagulant agents.



19 **20**

Conclusions and Future Directions- The results of ongoing clinical trials of novel thrombin inhibitors, the exploration of the applications of oral thrombin inhibitors and the development of therapeutically useful, low molecular weight competitive factor Xa inhibitors are anticipated in the very near future. These novel agents hold promise to change the practice of anticoagulant therapy.

References

1. R.L. Mueller and S.S. Scheidt, Circulation, 89, 432 (1994).
2. E.J. Topol, Am. J. Cardiol., 75, 27B (1995).
3. J.-P.R. Herrman and P.W. Serruys, Texas Heart Inst. J., 21, 138 (1994).
4. J. Hirsh and V. Fuster, Circulation, 89, 1449 (1994).
5. C. Tapparelli, R. Metternich, C. Ehrhardt and N.S. Cook, Trends Pharm. Sci., 14, 366 (1993).
6. J. Lefkovits and E.J. Topol, Circulation, 90, 1522 (1994).
7. F. A. Ofosu, G Agnelli and J. Hirsh, Recent Adv. Haematol., 6, 45 (1992).
8. S. Okamoto, K. Kinjo, A. Hijikata, J. Med. Chem., 23, 827 (1980).
9. H.K. Gold, F.W. Torres, H.D. Garabedian, W. Werner, I.-K. Jang, A. Khan J.N. Hagstrom, T. Yasuda, R.C. Leinbach, J.B. Newell, E.G. Bovill, D.C. Stump and D. Collen, J. Am. Coll. Cardiol., 21, 1039 (1993).
10. J. T. Willerson, and W. Casscells, J. Am. Coll. Cardiol., 21, 1048 (1993).
11. F. Markwardt, Thromb. Haemost., 66, 141 (1991).
12. T.J. Rydel, K.G. Ravichandran, A. Tulinsky, W. Bode, R. Huber, C. Roitsch, and J.W. Fenton II, Science, 249, 277 (1990).
13. F. Markwardt, Thromb. Res., 74, 1 (1994).
14. M. Verstraete, M. Nurmohamed, J. Kienast, M. Siebeck, G. Silling-Engelhardt, H. Buller, B. Hoet, J. Bichler, and P. Close, J. Am Coll. Cardiol., 22, 1080 (1993).
15. F. Schiele, A. Vuillemenot, Ph. Kramarz, Y. Kieffer, J. Soria, A. Camez, M.C. Mirshahi and J.P. Bassand, Thromb. Haemost., 71, 558 (1994).
16. A.A. van den Bos, J.W. Deckers, G.R. Heyndrickx, G.-J. Laarman, H. Suryapranata, F. Zijlstra, P. Close, J.J.M.M. Rijnierse, H.R. Buller and P.W. Serruys, Circulation, 88, 2058 (1993).
17. C.P. Cannon, C.H. McCabe, T.D. Henry, M.J. Schweiger, R.S. Gibson, H.S. Mueller, R.C. Becker, N.S. Kleiman, J.M. Haugland, J.L. Anderson, B.L. Sharaf, S.J. Edwards, W.J. Rogers, D.O. Williams and E. Braunwald, J . Am. Coll. Cardiol., 23, 993 (1994).
18. L.V. Lee, C.H. McCabe, E.M. Autman, M. Koch, R. Wilensky, K. Stringer, J. Hochman, H.S. Mueller, T.D. Henry, N. Kleiman, .M. Steingart, and H. Wasserman, J. Am. Coll. Cardiol. (Special Issue), 344a (1994).
19. The Global Use of Strategies to Open Occluded Coronary Arteries (Gusto) IIa Investigators, Circulation, 90, 1631 (1994).
20. E.M. Antman, for the TIMI 9A Investigators, Circulation, 90, 1624 (1994).
21. K.-L. Neuhaus, R.V. Essen, U. Tebbe, A. Jessel, H. Heinrichs, W. Mäurer, W. Döring, D. Harmjanz, V. Kötter, E. Kalhammer, H. Simon, and T. Hornacek, Circulation, 90, 1638, (1994).
22. B.E. Sobel, Circulation, 90, 2147 (1994).
23. J.M. Maraganore, P. Bourdon, J. Jablonski, K.L. Ramachandran, and J.W. Fenton II, Biochemistry, 29, 7095 (1990).
24. E.Skrzypczak-Jankun, V.E.Carperos, K.G.Ravichandran, A.Tulinsky, M. Westbrook, and J.M. Maraganore, J. Mol. Biol., 221,1379 (1991).
25. J.M. Maraganore in "The Design of Synthetic Inhibitors of Thrombin," G. Claeson, Ed., Plenum Press, New York, N.Y., 1993, p. 227.
26. R.-M. Lidón, P. Théroux, M. Juneau, B. Adelman, and J. Maraganore, Circulation, 88, 1495 (1993).
27. C.P. Cannon, J.M. Maraganore, J. Loscalzo, A. McAllister, K. Eddings, D. George, A.P. Selwyn, B. Adelman, I. Fox, E. Braunwald, and P. Ganz, Am. J. Cardiol., 71, 778 (1993).
28. E.J. Topol, R. Bonan, D. Jewitt, U. Sigwart, V.V. Kakkar, M. Rothman, D. De Bono, J. Ferguson, J.T. Willerson, J. Strony,P. Ganz, M.D. Cohen, R. Raymond, I.Fox, J. Maraganore, and B. Edelman, Circulation, 87, 1622 (1993).
29. J. Strony, J. A. Bittl, E. Deutsch, R. Ivanhoe, C. Meckel, B. Adelman, and the Hirulog Angioplasty Investigators, J. Am. Coll. Cardiol., Special Issue, 356A, 1006-43 (1995).
30. D.W. Banner and P. Hadváry, J. Biol. Chem., 266, 20085 (1991).
31. W. Bode, I Mayr, U. Baumann, R. Huber, S.R. Stone, and J. Hofsteenge, EMBO J., 8, 3467 (1989).
32. C. Kettner, and E. Shaw, Thromb. Res., 14, 969 (1979).
33. C.V. Jackson, V.G. Growe, J.D. Frank, H.C. Wilson, W.J. Coffman, B.G. Utterback, J.A. Jakubowski, and G.F. Smith, J. Pharmacol. Exp. Ther., 261, 546 (1992).

34. C.V. Jackson, H.C. Wilson, V.G. Growe, R.T. Shuman, and P.D. Gesellchen, J. Cardiovasc. Pharm., 21, 587 (1993).
35. M. Simoons, T. Lenderink, M. Scheffer, I. Stoel, I. P de Milliano, W. Remme, H. Werner, C. Leeners G. Molloek, A. Arnold, J. Stiibe, and E. Roberts, Circulation, 90, I-231 (1994).
36. R.T. Shuman, R.B. Rothenberger, C.S. Campbell, G.F. Smith, D.S. Gifford-Moore, and P.D. Gesellchen, J. Med. Chem., 36, 314 (1993).
37. W.E. Rote, E.M. Dempsey, G.L. Oldeshulte, R.J. Ardecky, S.Y. Tamura, R.F. Nutt, P.W. Bergum, H.S. Tran, S.M. Anderson and G.P. Vlasuk, Circulation, 90, I344 (1994).
38. N. Balasubramanian, D.R. St. Laurent, M.E. Federicic, N.A. Meanwell, J.J. Wright, W.A. Schumacher, and S.M. Seiler, J. Med. Chem., 36, 300 (1993).
39. B. Neises, and C. Tamus, Thromb. Haemost., 65, 1290 (1991).
40. T.R. Webb, T.A. Miller and G.P. Vlasuk, U.S. Patent # 5,371,072 (1994).
41. C. Kettner, L. Mersinger, and R. Knabb, J. Biol. Chem., 265, 18289 (1990).
42. C. Tapparelli, M. Powling, P. Gfeller, and R. Metternich, Thromb. Haemost., 65, 774 (1991).
43. C. Tapparelli, R. Metternich, C. Ehrhardt, M. Zurino, G. Claeson, M.F. Scully, and S.R. Stone, J. Biol. Chem., 268, 4734 (1993).
44. M. M. Abelman, D.A. Pearson, G.P. Vlasuk, and T.R. Webb, European Patent Application, WO 94/21673 (1994).
45. K. Hilpert, J. Ackermann, D.W. Banner, A. Gast, K. Gubernator, P. Hadváry, Ludvik, Labler, K. Müller, G. Schmid, T.B. Tschopp, H. Van de Waterbeemd, J. Med. Chem., 37, 3889 (1994).
46. A. Gast, T.B. Tschopp, G. Schmid, K. Hilpert, and J. Ackermann, Blood Coag. Fibrinol., 5, 879 (1994).
47. A. Gast, T.B. Tschopp, and H.R. Baumgartner, Arterioscler. Thromb., 14, 1466 (1994).
48. A.-C. Teger-Nilsson, U. Eriksson, D. Gustafsson, R. Bylund, G. Fager, and P. Held, J. Am. Coll. Cardiol. Special Issue, 117A (1995).
49. P. Zoldhelyi, J. Bichler, W.G. Owen, D.E. Grill, V. Fuster, J.S. Mruk and J.H. Chesebro, Circulation, 90, 2671 (1994).
50. K.G. Mann, M.E. Nesheim, W.R. Church, P. Haley and S. Krishnaswamy, Blood, 76, 1 (1990).
51. P.R. Eisenberg, J.E. Seigel, D.R. Abendshein, and J.P. Miletich, J. Clin. Invest., 91, 1877 (1993).
52. Lynch, J.J., Sitko, G.R., M.J. Mellott, E.M. Nutt, E.D. Lehman, P.A. Friedman, C.T. Dunwiddie, and G.P. Vlasuk, Cardiovasc. Res., 28, 78 (1994).
53. S. Hollenbach, U. Sinha, P.-H. Lin, K. Needham, L. Frey, T. Hancock, A. Wong, and D. Wolf, Thromb. Haemost., 71, 357 (1994).
54. C.R. Benedict, J. Ryan, J. Todd, K. Kuwabara, P. Tijburg, J. Cartwright Jr., and D. Stem, Blood, 81, 2059 (1993).
55. L.W. Schaffer, J.T. Davidson, G.P. Vlasuk, and P.K.S. Siegl, Circulation, 84, 1741 (1991).
56. M.E. Nesheim, C. Kettner, E. Shaw, and K.G. Mann, J. Biol. Chem., 256, 6537 (1981).
57. F.B. Taylor Jr, A.C.K. Chang, G.T. Peer, T. Mather, K. Blick, R. Catlett, M.S. Lockhart, and C.T. Esmon, Blood, 78, 364 (1991).
58. U. Sinha, T. Hancock, P.-H. Lin, S. Hollenbach, and D. Wolf, Prot. Exp. And Purif., 3, 518 (1992).
59. C.T. Dunwiddie, L. Waxman, G.P. Vlasuk, and P.A. Friedman, Methods Enzymol., 233, 291 (1993).
60. C.T. Dunwiddie, N.A. Thornberry, H.G. Bull, M. Sardana, P.A. Friedman, J.W. Jacobs, and E. Simpson, J. Biol. Chem., 264, 16694 (1989).
61. L. Waxman, D.E. Smith, K.E. Arcuri, and G.P. Vlasuk, Science, 248, 593 (1990).
62. G.P. Vlasuk, D. Ramjit, T. Fujita, C.T. Dunwiddie, E.M. Nutt, D.E. Smith, and R.J. Shebuski, Thromb. Haemost., 65, 257 (1991).
63. G.P. Vlasuk, Thromb. Haemost. 70, 212 (1993).
64. S. Krishnaswamy, G.P. Vlasuk, and P.W. Bergum, Biochemistry, 33, 7897 (1993).
65. G. Claeson, Blood Coag. Fibrinol., 5, 411 (1994).
66. S. Katakura, T. Nagahara, T. Hara, and M. Iwamoto, Biochem. Biophys. Res. Commun., 197, 965 (1993).
67. T. Nagahara, Y. Yokoyama, K. Inamura, S. Katakura, S. Komoriya, T. Yamaguchi, T. Hara, and M. Iwamoto, J. Med. Chem., 37, 1200 (1994).
68. T. Hara, A. Yokoyama, H. Ishihara, Y. Yokoyama, T. Nagahara, and M. Iwamoto, Thromb. Haemost., 71, 314 (1994).
69. G.P. Vlasuk, T.R. Webb, and D.A. Pearson, European Patent Application, WO 93/15756 (1993).
70. T.K. Brunck, T.R. Webb, and W. Ripka, European Patent Application, WO 94/13693 (1994).

Chapter 9. Potassium Channel Openers

James R. Empfield and Keith Russell
Department of Medicinal Chemistry, ZENECA Pharmaceuticals
Wilmington, DE 19897

Introduction - Potassium (K^+) channels play a central role in the regulation of cellular excitability. They are involved in setting the membrane potential, in addition to controlling the frequency and shape of action potentials. Tremendous advances have taken place in potassium channel science in the last few years. Recent reviews describing the role of K^+ channels in cell function, K^+ channel subtypes, molecular biology, biophysical and pharmacological classifications, K^+ channel blockers and openers, and clinical implications have been published (1-9). It is now clear that there are numerous types of potassium channel, each with a specific distribution and function in the body. Recent experiments have examined tissue-specific and developmental aspects of K^+ channel gene expression as well as the regulation of K^+ channel gene expression by growth factors, hormones, and depolarization (10). Site directed mutagenesis has been used on both the potassium channel and its ligand (in this case charybdotoxin) to provide three dimensional information of the contact surface between the two proteins (11). The first clear images of a voltage-gated (shaker) ion channel, obtained by electron microscopy, have revealed a marked 4-fold symmetry to the channel, supporting earlier work that suggested a tetrameric nature for this type of channel (12).

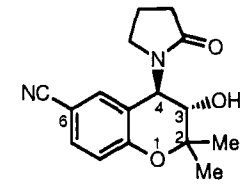
Activation or opening of potassium channels by organic molecules have been largely confined to the adenosine triphosphate (ATP)-sensitive (K_{ATP}) and large conductance calcium (Ca^{2+})-activated (BK_{Ca}) potassium channels (13-15). Reviews of the cellular pharmacology of potassium channel openers/activators (PCOs) in vascular smooth muscle (6), their mechanism of action (16), and their clinical potential (17) are available.

ATP-SENSITIVE POTASSIUM CHANNELS (K_{ATP})

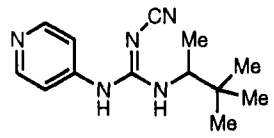
Several aspects of the K_{ATP} channel have recently been reviewed (18). During the past few years intense research has focused on the structure, function, and distribution of the target channel for the prototypical PCOs, cromakalim and pinacidil. Adelman has reported the cloning and expression of an inward rectifier channel with many of the properties of the K_{ATP} channel, except it is not sensitive to the K_{ATP} sulfonyl urea blockers (19). This channel can exist and function independently of the sulfonylurea receptor. It is therefore likely that the K_{ATP} channel exists as a complex of two or more proteins (20). The sulfonyl urea receptor (also called the glyburide binding protein) (21), a member of the ATP binding cassette/transporter group of proteins, has been cloned and expressed (22). No reports of the coexpression of the cloned inward rectifier channel with the glyburide binding protein have been reported to date. Activators of K_{ATP} channel may exert their effect by competing with ATP (23). It has recently been suggested that the K_{ATP} channel is a voltage-insensitive state of the delayed rectifier channel, K_v (24). Recent evidence, suggests that the PCOs may possess further mechanisms of vasorelaxation not linked to the opening of plasmalemmal K^+ channels (25,26).

K_{ATP} PCOs: Structural Classes - Structure-activity relationships (SAR) of the structurally diverse K_{ATP} PCOs have been reviewed (27,28). The structural classes of K_{ATP} PCOs and their SAR have expanded over the last few years. However, the distinction between some classes has diminished with the development of common pharmacophore models for PCOs leading to new compounds with structural features which span the different classes (29-31). Nonetheless, most PCOs broadly fit into eight structural classes: [class (prototypical PCO)]; benzopyrans (cromakalim, **1**), cyanoguanidines (pinacidil, **3**), thioformamides (aprikalim, **4**), tertiary carbinols (**5**), dihydropyridines (**6**), pyridyl nitrates (nicorandil, **7**), benzothiadiazines (diazoxide, **8**), and pyrimidine sulfates (minoxidil sulfate, **9**). The benzopyran class continues to receive the most attention while the tertiary carbinol series represents the newest structural class.

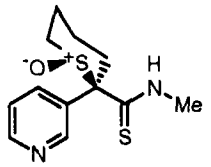
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.



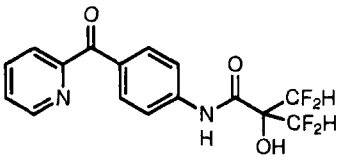
1
2



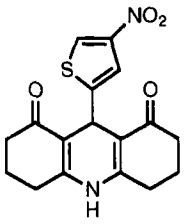
3



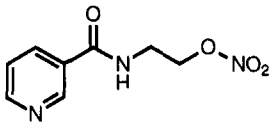
4



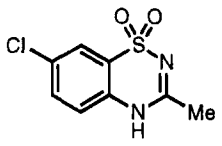
5



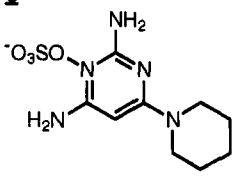
6



7



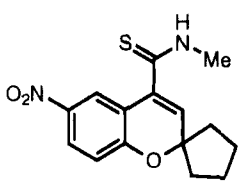
8



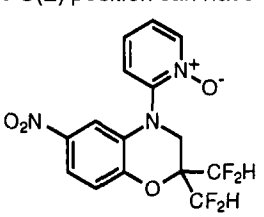
9

Stereochemistry: Racemic: (▬) Single Enantiomer: (◀)

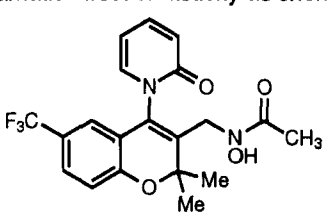
Benzopyrans (Cromakalim) - The initial modest changes around cromakalim have yielded to drastic structural variation within this class over the past few years. First generation modifications concentrated on replacements of the C(4) pyrrolidinone of cromakalim. This subject has previously been reviewed, therefore only new developments will be highlighted (28,32). Modest changes at the C(2) position can have dramatic effect on activity as exem-



10

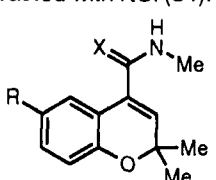


11



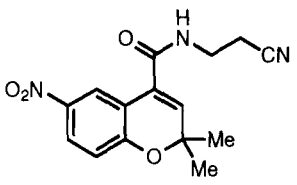
12

plified by 10, an analog reported to be ~10,000 times more potent *in vitro* (rat aorta) than cromakalim, RP 49356 (±)-4), and pinacidil (33). The tetrafluorobenzoxazine 11 is 80 fold more potent than cromakalim in relaxing guinea pig detrusor strips contracted with KCl (34). 2-Alkoxy substituted chromans and benzoxazines derivatives have been claimed as PCOs in recent patent applications (35,36). At the C(3) position the N-acetyl hydroxylamine analog 12 has been claimed to possess both PCO (pIC_{50} = 8.72; 20 mM KCl precontracted rat aorta) and 5-lipoxygenase inhibitor activities (pIC_{50} = 4.3; A23187-induced stimulation of leukotriene B_4 in human whole blood) (37). Variations at the C(4) position include acyclic amides, thioamides, cyanoguanidines, cyclic sulfoxides, imidazolones, and 2-nitropyrroles. Derivatives 13 and 14 were found to have comparable *in vitro* (30 mM KCl contracted rat aorta) activity to cromakalim (31). The thioamide

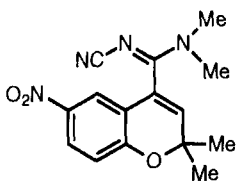


13 X=O, R=CN
14 X=NCN, R=CN
15 X=S, R=CN
22 X=S

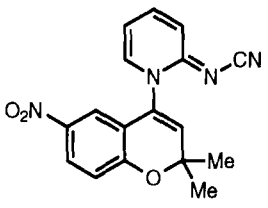
derivative **15** however is nearly tenfold more potent than cromakalim in this assay and on inhibiting the spontaneous tone in guinea pig tracheal spirals (31,38). N-Substituted carboxamides such as **16** are even more potent analogs in the rat aorta assay (39). A number of ureas (40), N-cyanoamidines (41), and cyanoguanidines (42) have been employed as isosteres for the cyclic amides in the benzopyran PCOs. Unlike cromakalim, **17** displays a nearly 1000-fold tissue selectively for guinea-pig trachealis vs. rat aorta (41). A related derivative, SR47063 (**18**) is significantly more potent than cromakalim or bimakalim



16

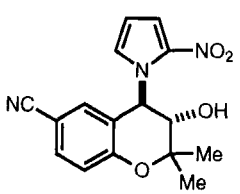


17

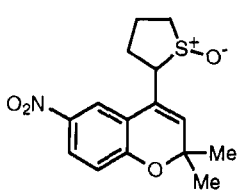


18

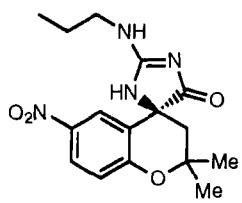
in patch-clamp studies (43). More dramatic changes in C(4) isosteres have led to the 2-nitro pyrrole **19** (44), sulfoxide **20** (45), and spiroimidazolone **21** (46) analogs. The spirocycle of **21** constrains the plane of the heterocycle orthogonal to the benzopyran. This rigid analog is significantly more potent than cromakalim both *in vitro* and *in vivo* (rat hypotensive activity). Additionally, the S-isomer (**21**) is 100 times more potent than the R-isomer(47). A comparative molecular field analysis (48) and quantitative structure-activity relationship (QSAR) study (49) on 6-substituted benzopyran-4-carbothioamides (**22**) indicate that the vasorelaxant activity is linearly correlated with the electron parameter, σ, and parabolically with steric and hydrophobic, π, parameters. The order of potency is $NO_2 > CF_3 > OCF_3 > Br > CN$.



19

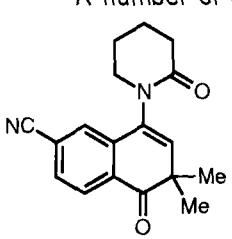


20

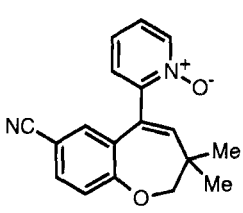


21

A number of benzopyran nucleus modifications have been reported. 2,2-Dialkylnaphthalen-1-ones such as UR-8225 (**23**) have been reported to have similar activity as the benzopyran series *in vitro* in isolated rat portal vein and guinea pig tracheal spirals (50,51). Benzocycloheptenes, benzoxepines (**24**) (EC_{25} = 0.7 μM; ^{86}Rb efflux in guinea pig trachea) and benzothiepines have recently been claimed as novel PCOs (52).

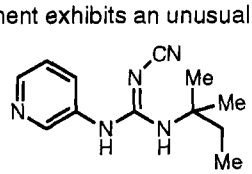


23



24

Cyanoguanidines (Pinacidil) - The X-ray crystal structure of pinacidil has been determined and indicated that the N',N"-disubstituted N-cyanoguanidine fragment exhibits an unusual (Z,Z)-conformation (53). A related analog, P1075 (**25**) has been radiolabeled and found to exhibit specific binding in rat aortas at concentrations lower than required for K^+ channel opening (54). Furthermore K_{ATP} PCOs from the pinacidil, cromakalim, aprikalim, diazoxide, and minoxidil classes as well as the specific blocker , glyburide were all found to be capable of displacing [^{3}H]-P1075.

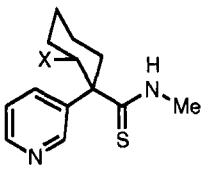


25

26

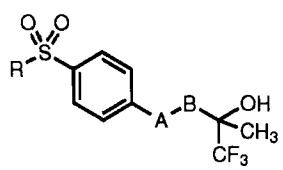
The authors suggest that PCOs from these different classes interact with the same target protein but different allosterically coupled receptor binding sites (55). A related analog from this series, AL0670 (**26**), has been chosen for development as an antihypertensive agent (56). Although structurally similar, it possesses a different pharmacological and conformational profile from pinacidil.

Thioformamides (Aprikalim) - A series of potent PCOs exemplified by RP66266 (**27**) and RP66784 (**28**) antagonized 20 mM KCl-induced contractions in rat aorta with IC_{90}s of 3.0 and 0.3 nM respectively (57).

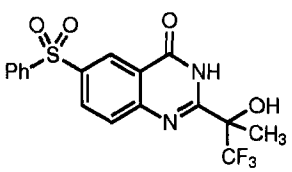


27 X= (=$CHCH_2SPh$
28 X= $CH_2CH_2NHSO_2Ph$

Tertiary Carbinols - A new series of agents, the tertiary carbinols, was recently described including several *in vivo* bladder selective agents (58,59). A strategy involving the careful manipulation of conformation and pKa in the amide tertiary carbinols series led to **5** which was an order of magnitude more potent than cromakalim in a guinea-pig detrusor assay (60). Structural variants incorporating olefin (**29**)(61), ether (**30**)(61), and heterocyclic replacements (**31**)(62), for the amide linker moiety retain significant PCO activity. Replacement of the aryl sulfonyl and benzoyl moieties by sulfonamides has yielded active PCOs (**32**) (63).

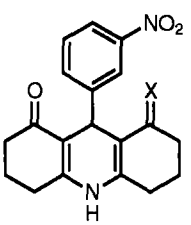


29 A-B=*t*-CH=CH, R=Ph
30 A-B=O-CH_2, R=Ph
32 A-B=NH-CO, R=NEt_2

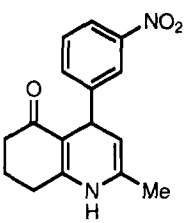


31

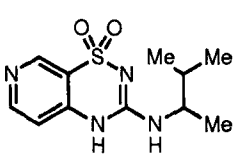
Dihydropyridines - The dihydropyridine system is usually associated with calcium L-channel blockade and activation. Recently, three related series of dihydropyridine K_{ATP} PCOs (**33**-**35**) have been described. Unlike niguldipine which has negligible activity on K^+ (15mM) precontracted guinea-pig detrusor, these analogs show significant K_{ATP} PCO activity. The thiophene analog, **6** is three times more potent than cromakalim in this assay (64).



33 X=O
34 X=H, H



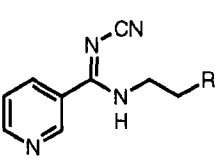
35



36

Benzothiadiazines (Diazoxide) - A series of pyridothiadiazines, exemplified by BPDZ 44 (**36**), showing a partial structural analogy with the two PCOs diazoxide and pinacidil, inhibit the insulin releasing process by activating K_{ATP} (65). BPDZ 44 was found to be more potent than diazoxide at inhibiting insulin secretion from rat pancreatic ß-cells (66).

Pyridyl nitrates (Nicorandil) - The mechanism of action of KRN2391 (**37**) and nicorandil is dependent on their site of action. Each shows a nitrate action alone in large canine coronary artery, and increasing PCO action as the diameter of the coronary artery decreases (67-70). The vasodilatory effects of a related analog, Ki3315 (**38**) are predominantly due to a K_{ATP} PCO mechanism (71). The vasorelaxant mechanism of Ki1769 (**39**), comprising a hybrid between nicorandil and pinacidil, is also via opening of K_{ATP} (72).

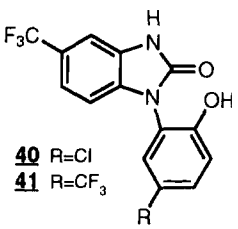


37 R=ONO_2 ($MeSO_3H$ salt)
38 R=OH
39 R=Ph

LARGE CONDUCTANCE CALCIUM-DEPENDENT POTASSIUM CHANNELS (BK_{Ca})

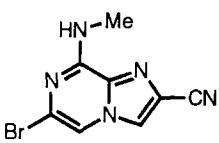
The primary structure of the α-subunit of the cloned human BK_{Ca} channel shows close structural homology to the voltage-gated potassium channels. It is thus likely to be a six membrane spanning helical protein (73). Recently, a ß- subunit has been cloned that is important for modulation of the channel by openers (74).

A recent review of BK_{Ca} openers has been published (75). The most studied BK_{Ca} activators are NS 004 (**40**) and NS 1619 (**41**) (76-78). Although NS 004 produced a significant increase in the activity of single GH3 cell BK_{Ca} channels and in rat brain BK_{Ca} channels reconstituted into planar lipid bilayers (79), the compound is not selective for this channel subtype (80-82). NS 1619 also activates BK_{Ca} channel (83), however, NS 1619 inhibits both K_V and L-type Ca^{2+} channels over the same concentration range it activates the BK_{Ca} (84).



40 R=Cl
41 R=CF_3

The most potent BK_{Ca} openers discovered to date are the soyasaponins, which were isolated from a medicinal herb used in Ghana as a treatment for asthma (85). These compounds act on the cytoplasmic face of the channel. The ß-subunit of BK_{Ca} confers sensitivity to these openers (86).

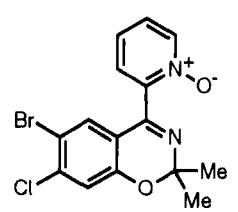


42

SCA40 (**42**) relaxes vascular smooth muscle *in vitro* and *in vivo*. These effects are claimed to involve activation of K^+-channels distinct from glyburide-sensitive K_{ATP} channels (87,88). Definitive proof of the mode of action and selectivity of SCA40 is not available. At subnanomolar concentrations, Pb^{2+} activates a BK_{Ca} channel in bovine adrenal chromaffin cells (89). BK_{Ca} channels are rapidly activated by niflumic acid dose-dependently and reversibly. External niflumic acid is about 5 times more potent than internal niflumic acid (90). Nitric oxide/EDRF directly activates BK_{Ca} in rabbit aorta (91).

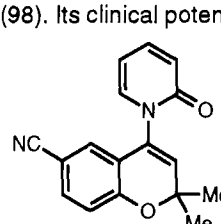
THERAPEUTIC INDICATIONS

Cardiovascular - A recent review describes the utility of PCOs in treating hypertension (92). Some of the newer drugs may offer special advantages in the presence of concomitant diseases, such as diabetes mellitus, hyperlipidemia, angina pectoris or congestive heart failure (92). Reflex tachycardia and vasodilator headache continue to limit the utility of these agents as antihypertensive agents. The antihypertensive effects induced by TCV-295 (**43**) were studied in rats and dogs. It possesses a longer duration of action than levcromakalim and the Ca^{2+} channel blocker nisoldipine, and the reflex tachycardia it evoked was less marked (93).



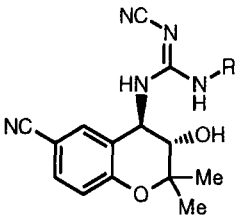
43

ATP-sensitive PCOs are of potential benefit in ischemic heart disease (94-96). Nicorandil combines the pharmacological properties of an organic nitrate with those of a potassium channel activator. It was launched in Japan for the treatment of angina in 1984 (98). Its clinical potential has been reviewed (95,97,98). A part of the antianginal efficacy of nicorandil may be the result of its ability to increase coronary collateral blood flow (99). Nicorandil, aprikalim, and bimakalim (**44**) are protective in two experimental models of ischemia/reperfusion injury. The mechanism of action of these agents in this model is not completely understood, but it appears to be a result of myocardial K_{ATP} channel opening (100-104). Bimakalim treatment prior to the onset of a 60-min coronary occlusion increases the tolerance of pig myocardium to ischemia (105). Myocardial infarct size was also

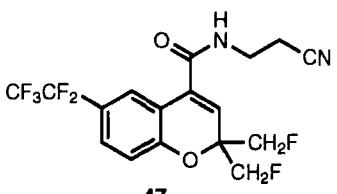


44

significantly ($P < .05$) reduced (38%) by aprikalim and significantly increased (38%) by the K_{ATP} blocker glyburide (106). Recent reports have described cardioselective PCOs. Thus benzo-pyranylcyanoguanidines including BMS-180448 (**45**) are cardioselective anti-ischemic K_{ATP} PCOs with no correlation between vasorelaxant and anti-ischemic potencies (27,107,108). In addition, these anti-ischemic effects can be obtained at significantly lower concentrations than those required to reduce the action potential duration (APD) (109,110). Another cromakalim analog, U-89232 (**46**) produces myoprotection that is not reversed by glyburide (111). U-89232 is apparently cardioselective since it exhibits myoprotection without producing hypotension (112,113).



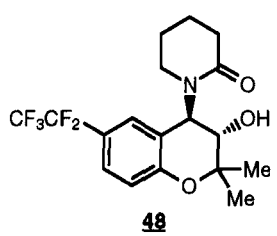
45 R=*p*-Cl-C_6H_4
46 R=$C(Me)_2CH_2CH_3$



47

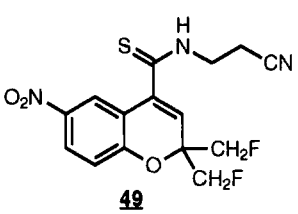
The favorable effects of some PCOs on lipid profiles has been noted recently. Thus AL0671 (HCl salt of **26**) activates both lipoprotein lipase and hepatic triglyceride lipase activities through its PCO activity and decreases triglyceride-rich lipoproteins in genetically obese hyperlipidemic rats (114). The effects of KC-515 (**47**) in several hypertensive and hyperlipidemia models of rats are outlined in a recent paper (115).

Asthma - Several reviews have explored the prospects for potassium channel activators in the treatment of airway obstruction and hyper-reactivity, and their potential utility in asthma (116-120). Recent studies have suggested the possibility that bronchodilator ß2-agonists exert their effects, at least in part, by opening potassium channels (121,122). A key challenge in this area is to find a PCO that exerts its effects on the airways at doses that do not cause cardiovascular effects. Most of the studies to date have been with K_{ATP} PCOs.



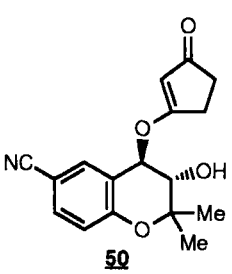
48

BRL 55834 (**48**), an analog of cromakalim, is claimed to show some selectivity for the lung (123). It is twice as potent as levcromakalim and one sixth as potent as salbutamol in delaying the onset of dyspnea in response to inhaled histamine. It was effective at the earliest time studied, but the peak effect of BRL 55834 tended to be delayed and it was longer acting than levcromakalim or salbutamol (124). The benzopyran, KC-399 (**49**) is an orally active, potent and long lasting bronchodilator (ED_{50}s being 0.03 µM for KC-399 vs 1.0 µM, for levcromakalim) (125,126). The vasorelaxation with KC-399 developed more slowly than levcromakalim and was more resistant to washout (127).



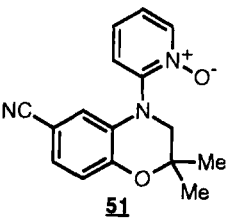
49

The realization that asthma is a chronic inflammatory disease accompanied by bronchial hypersensitivity towards a number of different stimuli, has led to a focus on the effects of PCOs on these parameters. In one study SDZ PCO-400 (**50**) was found to be modestly effective in resolving airway obstruction in normal animals (128). In contrast, there was marked resolution of airway obstruction in hyperreactive animals. Attempts to demonstrate an overt cellular anti-inflammatory activity of PCOs has been less successful. In a study with guinea pigs expressing idiopathic pulmonary eosinophilia, chronic administration of SDZ PCO-400 (1 mg/kg p.o. for 10 days) did not change bronchial or peritoneal eosinophil number (128). In general PCOs have little effect on cellular inflammation but may modulate neurogenic inflammatory

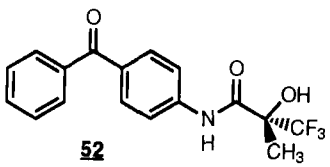


50

responses by inhibiting neuropeptide release from nerve terminals. This has been suggested as the basis behind the efficacy of cromakalim in the nocturnal asthma clinical trial (117,129). YM934 (**51**) inhibits airway neurogenic inflammation by modulating the release of neuropeptides from the sensory nerve endings (130). Levcromakalim (0.025-0.2 mg/kg i.v.) and aprikalim (0.05-0.5 mg/kg i.v.) inhibit bronchoconstrictor responses to excitatory non-adrenergic non-cholinergic (NANCe) nerve stimulation (131).



51



52

Urinary Incontinence - PCOs relax bladder smooth muscle precontracted with KCl (132,133). This has led to an interest in their application in treating urge incontinence(UI) which is often associated with an instability or hyperreactivity of the detrusor smooth muscle. A review describing the possible utility of PCOs for treating idiopathic detrusor instability has appeared (134). Selectivity for the bladder is an important requirement for clinically useful agents. Comparison of the effects of several PCOs on rat bladder and rat portal vein *in vitro* did not show evidence of tissue selectivity (132). Recently, bladder selective PCOs devoid of significant cardiovascular effects have been described. One agent, ZD6169 (**52**) from the tertiary carbinol series of PCOs may be of value as a treatment for urge incontinence (58,59). Members of a series of novel 4-indazolinonyl derivatives of benzopyrans may also have utility in treating UI (135,136).

Central Nervous System - The results that cromakalim and diazoxide can prevent the initial hyperexcitability produced by anoxia in rat hippocampal slices suggest that these drugs might be useful in the treatment of seizures (137). The anticonvulsant activity of cromakalim (injected centrally) against digoxin-induced convulsions (138), and that of other pyran PCOs in a rodent electroshock seizure model have been reported and shown to be antagonized by K^+ channel blockers (139).

Potassium channel openers are also being studied as novel therapeutics for the treatment of pain. The PCOs diazoxide, minoxidil, and levcromakalim produced antinociception in the rat tail flick model. This effect is antagonized by glyburide supporting K_{ATP} opening as the mode of action (140). The antinociception induced by morphine (a μ opioid agonist) is markedly potentiated by cromakalim. These findings suggest that the activation of central K_{ATP} channels may play an important role in the modulation of the antinociception induced by μ agonists (141).

The role of potassium channels in other CNS indications such as in Alzheimer's disease (142), psychosis (143), and anesthesiology (144) is being investigated.

Other Indications - Several structurally distinct PCOs cause hair growth (hypertrichosis). Recent studies suggest this may be related to a stimulation of DNA synthesis (145). The expression of K^+ channels modulates cell proliferation in a variety of tumor cells. Experimental evidence and three putative mechanisms of K^+ channel action on cell proliferation are discussed in a recent review (146). The effect of cromakalim on the growth of human brain tumor cells was investigated in a recent study (147). Cromakalim inhibited the growth of SK-N-MC human neuroblastoma and U-373 MG human astrocytoma cell lines in a dose-dependent manner. This effect of cromakalim was significantly blocked by the co-treatment with the K_{ATP} blockers, glyburide or tolbutamide (147).

Summary - The science of potassium channels is in a very exciting phase. New potassium channels are being cloned, and our knowledge of structure and function of K^+ channels is rapidly increasing. Structurally diverse series of PCOs continue to be discovered. A large number of therapeutic targets are being investigated with this class of agents. Tissue selectivity will undoubtedly play a key role in their success.

References

1. A. L. Harvey, Med. Res. Rev., 13, 81 (1993).
2. L. Salkoff, K. Baker, A. Butler, M. Covarrubias, M. D. Pak and A. Wei, TINS, 15, 161 (1992).
3. C. T. Bond, M. Pessia, X-M. Xia, A. Lagrutta, M. P. Kavanaugh and J. P. Adelman, Recept. Channels, 2, 183 (1994).
4. T. Hoshi and W. N. Zagotta, Curr. Opin. Neurobiol., 3, 283 (1993).
5. J. O. Dolly, J. Rettig, V. E. S. Scott, D. N. Parcej, R. Wittkat, S. Sewing and O. Pongs, Biochem. Soc. Trans., 22, 473 (1994).
6. U. Quast, J-M. Guillon and I. Cavero, Cardiovasc. Res., 28, 805 (1994).
7. S. C. Hebert and K. Ho, Renal Physiol. Biochem., 17, 143 (1994).
8. P. N. Strong, Endocrinol. Metab., 6, 373 (1994).
9. G. Prestipino, M. Nobile and E. Maestrone, Endocrinol. Metab., 6, 261 (1994).
10. T. M. Perney and L. K. Kaczmarek, Semin. Neurosci., 5, 135 (1993).
11. M. Stocker and C. Miller, Proc. Natl. Acad. Sci. U. S. A., 91, 9509 (1994).
12. M. Li, N. Unwin, K. A. Stauffer, Y. N. Jan and L. Y. Jan, Curr. Biol., 4, 110 (1994).
13. K. S. Atwal, Med. Res. Rev., 12, 569 (1992).
14. R. H. Poyser and T. C. Hamilton, Drugs of the Future, 19, 39 (1994).
15. M. T. Nelson, Trends. Cardiovasc. Med., 3, 54 (1993).
16. G. A. McPherson, Gen. Pharmacol., 24, 275 (1993).
17. U. Quast, K. M. Bray, Y. Baumlin and J. Dosogne, Pharmacochem. Libr., 18, 309 (1992).
18. U. Panten, C. Schwanstecher and M. Schwanstecher, Handb. Exp. Pharmacol., 108, Pt. 2, 547 (1993).
19. M. L. J. Ashford, C. T. Bond, T. A. Blair and J. P. Adelman, Nature (London), 370, 456 (1994).
20. R. N. Khan, C. N. Hales, S. E. Ozanne, A. A. Adogu and M. L. J. Ashford, Proc. R. Soc. London, Ser. B, 253, 225 (1993).
21. H. Bernardi and M. Lazdunski, Methods Pharmacol., 7, 61 (1993).
22. L. Aguilar-Bryan, C. G. Nichols, S. W. Wechsler, J. P. Clement IV, A. E. Boyd III, G. Gondalez, H. Herrera-Sosa, K. Nguy, B. Bryan, and D. A. Nelson, Science , 268, 423 (1995).
23. P. Henry and D. Escande, Cardiovasc. Res., 28, 754 (1994).
24. G. Edwards and A. H. Weston, Br. J. Pharmacol., 110, 1280 (1993).
25. U. Quast, Trends Pharmacol. Sci., 14, 332 (1993).
26. K. Lawson and P. E. Hicks, Curr. Opin. Invest. Drugs, 2, 1209 (1993).
27. K. S. Atwal, J. Cardiovasc. Pharmacol., 24, S12 (1994).
28. J. M. Evans and S. G. Taylor, Prog. Med. Chem., 31, 411 (1994).
29. K. S. Atwal, S. Moreland, J. R. McCullough, B. C. O'Reilly, S. Z. Ahmed and D. E. Normandin, Bioorg. Med. Chem. Lett., 2, 83 (1992).
30. K. S. Atwal, Drug Dev. Res., 33, 250 (1994).
31. H. Koga, M. Ohta, H. Sato, T. Ishizawa and H. Nabata, Bioorg. Med. Chem. Lett., 3, 625 (1993).
32. J. M. Evans and S. D. Langman, Annual Reports in Medicinal Chemistry, 26, 73 (1991).
33. H. Sato, H. Koga, T. Ishizawa, T. Makino, K. Kuromaru, N. Taka, T. Takahashi, T. Sato and H. Nabata, Bioorg. Med. Chem. Lett., 3, 2627 (1993).
34. K. Russell, F. J. Brown, P. Warwick, J. Forst, T. Grant, B. Howe, S. T. Kau, J. H. Li and F. M. McLaren, Bioorg. Med. Chem. Lett., 3, 2727 (1993).
35. M. Covarrubias, A. Wei, L. Salkoff and T. B. Vyas, Neuron, 13, 1403 (1994).
36. Y. Matsumoto, W. Uchida and I. Yanagisawa, Patent Application, WO/9418195 (1994).
37. A. W. Lochead, M. J. Navet and P. E. Hicks, Patent Application, EP 529654 (1993).
38. J. R. S. Arch, D. R. Buckle, C. Carey, H. Parr-Dobrzanski, A. Faller, K. A. Foster, C. S. V. Houge-Frydrych, I. L. Pinto, D. G. Smith and S. G. Taylor, J. Med. Chem., 34, 2588 (1991).
39. H. Koga, H. Sato, T. Ishizawa, K. Kuromaru, T. Makino, N. Taka, T. Takahashi, T. Sato and H. Nabata, Bioorg. Med. Chem. Lett., 3, 1115 (1993).
40. G. Burrell, J. M. Evans, F. Hicks and G. Stemp, Bioorg. Med. Chem. Lett., 3, 999 (1993).
41. T. Ishizawa, H. Koga, H. Sato, T. Makino, N. Taka, T. Takahashi, T. Sato and H. Nabata, Bioorg. Med. Chem. Lett., 4, 1995 (1994).
42. K. S. Atwal, S. Moreland, J. R. McCullough, S. Z. Ahmed and D. E. Normandin, Bioorg. Med. Chem. Lett., 2, 87 (1992).
43. Y. Tourneur, A. Marion and P. Gautier, J. Membr. Biol., 142, 337 (1994).
44. D. G. Smith, D. R. Buckle, A. Faller and I. L. Pinto, Bioorg. Med. Chem. Lett., 2, 1595 (1992).
45. S. E. Yoo, S. J. Kim, H. S. Shin and K. W. Hong, Bioorg. Med. Chem. Lett., 3, 553 (1993).
46. R. C. Gadwood, B. V. Kamdar, L. A. C. Dubray, M. L. Wolfe, M. P. Smith, W. Watt, S. A. Mizsak and V. E. Groppi, J. Med. Chem., 36, 1480 (1993).
47. R. C. Gadwood, L. M. Thomasco, V. E. Groppi, B-A. Burrnett, S. J. Humphrey, M. P. Smith and W. Watt, 207th ACS National Meeting, San Diego, CA, March 13-18, Abstract 223, (1994).
48. M. Ohta, H. Koga, H. Sato and T. Ishizawa, Bioorg. Med. Chem. Lett., 4, 2903 (1994).
49. T. Ishizawa, H. Koga, M. Ohta, H. Sato, T. Makino, K. Kuromaru, N. Taka, T. Takahashi, T. Sato and H. Nabata, Bioorg. Med. Chem. Lett., 3, 1659 (1993).
50. C. Almansa, L. A. Gomez, F. L. Cavalcanti, R. Rodriguez, E. Carceller, J. Bartroli, J. Garcia-Rafanell and J. Forn, J. Med. Chem., 36, 2121 (1993).
51. F. Perez-Vizcaino, O. Casis, R. Rodriguez, L. A. Gomez, J. Garcia Rafanell and J. Tamargo, Br. J. Pharmacol., 110, 1165 (1993).
52. J-J. Berthelon, M. Brunet, M. Noblet, P. Durbin, D. Guerrier and T. Luong, Patent Application, WO/

9413658 (1994).
53. B. Pirotte, L. Dupont, P. de Tullio, B. Masereel, M. Schynts and J. Delarge, Helv. Chim. Acta, 76, 1311 (1993).
54. U. Quast, K. M. Bray, H. Andres, P. W. Manley, Y. Baumlin and J. Dosogne, Mol. Pharmacol., 43, 474 (1993).
55. P. W. Manley, U. Quast, H. Andres and K. Bray, J. Med. Chem., 36, 2004 (1993).
56. M. Eda, T. Takemoto, S. Ono, T. Okada, K. Kosaka, M. Gohda, S. Matzno, N. Nakamura, C. Fukaya, J. Med. Chem. 37, 1983 (1994).
57. T. J. Brown, R. F. Chapman, J. S. Mason, M. N. Palfreyman, N. Vicker and R. J. A. Walsh, J. Med. Chem., 36, 1604 (1993).
58. T. L. Grant, C. J. Ohnmacht and B. B. Howe, Trends Pharmacol. Sci., 15, 402 (1994).
59. J. R. Empfield, C. J. Ohnmacht, K. Russell, F. J. Brown, C. A. Frank, K. H. Gibson, T. Grant, T. J. Halterman, B. B. Howe, D. A. Trainor, S. T. Kau, F. M. McLaren, H. S. Shapiro, S. Trivedi, C. Yochim, J. H. Li and D. R. Mayhugh, 207th ACS National Meeting, San Diego, CA, March 13-18, Abstract 128 (1994).
60. T. Grant, C. A. Frank, S. T. Kau, J. H. Li, F. M. McLaren, C. J. Ohnmacht, K. Russell, H. S. Shapiro and S. Trivedi, Bioorg. Med. Chem. Lett., 3, 2723 (1993).
61. K. Russell, J. R. Empfield, C. J. Ohnmacht and K. H. Gibson, Patent Application, WO 93/23358 (1993)
62. K. Russell, Patent Application, EP 625511 (1994).
63. J. R. Empfield, D. R. Mayhugh and K. Russell, Patent Application, EP 625516 (1994).
64. C. A. Frank, J. M. Forst, T. Grant, R. J. Harris, S. T. Kau, J. H. Li, C. J. Ohnmacht, R. W. Smith, D. A. Trainor and S. Trivedi, Bioorg. Med. Chem. Lett., 3, 2725 (1993).
65. B. Pirotte, M. H. Antoine, P. de Tullio, M. Hermann, A. Herchuelz, J. Delarge and P. Lebrun, Biochem. Pharmacol., 47, 1381 (1994).
66. B. Pirotte, P. de Tullio, P. Lebrun, M. H. Antoine, J. Fontaine, B. Masereel, M. Schynts, L. Dupont, A. Herchuelz and J. Delarge, J. Med. Chem., 36, 3211 (1993).
67. Y. Fukata, S. Kaneta, Y. Okada, T. Yokoyama, Y. Jinno, H. Fukushima and N. Ogawa, Jpn. J. Pharmacol., 63, 305 (1993).
68. N. Ogawa, Gen. Pharmacol., 25, 609 (1994).
69. A. Miwa, Y. Jinno, T. Yokoyama, T. Izawa and N. Ogawa, Gen. Pharmacol., 25, 471 (1994).
70. T. Yokoyama, S. Kaneta, A. Miwa, Y. Tanaka, H. Isumi, T. Izawa and N. Ogawa, Arch. Int. Pharmacodyn. Ther., 327, 194 (1994).
71. Y. Tanaka, Y. Okada, T. Tokoyama, T. Nakajima, T. Izawa, H. Fukushima and N. Ogawa, Arch. Int. Pharmacodyn. Ther., 326, 62, (1993).
72. T. Kashiwabara, T. Nakajima, H. Kasai, S. Nakajima, T. Izawa and N. Ogawa, Arch. Int. Pharmacodyn. Ther., 327, 175 (1994).
73. S. I. Dworetzky, J. T. Trojnacki and V. K. Gribkoff, Mol. Brain Res., 27, 189 (1994).
74. H. G. Knaus, K. Folander, M. Garcia-Calvo, M. L. Garcia, G. J. Kaczorowski, M. Smith and R. Swanson, J. Biol. Chem., 269, 17274 (1994).
75. S-P. Olesen, Expert Opin. Invest. Drugs, 3, 1181 (1994).
76. S-P. Olesen, L. H. Jensen and P. Moldt, Patent Application, EP 617023 (1994).
77. S-P. Olesen, E. Munch, F. Watjen and J. Drejer, NeuroReport, 5, 1001 (1994).
78. S-P. Olesen, E. Munch, P. Moldt and J. Drejer, Eur. J. Pharmacol., 251, 53 (1994).
79. M. C. McKay, S. I. Dworetzky, N. A. Meanwell, S-P. Olesen, P. H. Reinhart, I. B. Levitan, J. P. Adelman and V. K. Gribkoff, J. Neurophysiol., 71, 1873 (1994).
80. X. Xu, T. D. Tsai, J. Wang, E. W. Lee and K. S. Lee, J. Pharmacol. Exp. Ther., 271, 362 (1994).
81. V. K. Gribkoff, G. Champigny, P. Barbry, S. I. Dworetzky, N. A. Meanwell and M. Lazdunski, J. Biol. Chem., 269, 10983 (1994).
82. K. Bian and K. Hermsmeyer, J. Vasc. Res., 31, 256 (1994).
83. A. J. Sellers and M. L. J. Ashford, Br. J. Pharmacol., 113, 659 (1994).
84. G. Edwards, A. Niederste-Hollenberg, J. Schneider, T. Noack and A. H. Weston, Br. J. Pharmacol., 113, 1538 (1994).
85. O. B. McManus, G. H. Harris, K. M. Giangiacomo, P. Feigenbaum, J. P. Reuben, M. E. Addy, J. F. Burka, G. J. Kaczorowski and M. L. Garcia, Biochemistry, 32, 6128 (1993).
86. O. B. McManus, L. Pallanck, L. M. H. Helms, R. Swanson and R. J. Leonard, 24th Annual Meeting of the Society for Neuroscience Abstracts, Abstract 20 (1994).
87. A. Michel, F. Laurent, J. Bompart, K. Hadj-Kaddour, J. P. Chapat, M. Boucard and P. A. Bonnet, Br. J. Pharmacol., 110, 1031 (1993).
88. F. Laurent, A. Michel, P. A. Bonnet, J. P. Chapat and M. Boucard, Br. J. Pharmacol., 108, 622 (1993).
89. L. R. Sun and J. B. Suszkiw, Neurosci. Lett., 182, 41 (1994).
90. M. Ottolia and L. Toro, Biophys. J., 67, 2272 (1994).
91. V. M. Bolotina, S. Najibi, J. J. Palacino, P. J. Pagano and R. A. Cohen, Nature (London), 368, 850 (1994).
92. P. A. van Zwieten, Clin. Exp. Hypertens., 15, 1205 (1993).
93. K. Kusumoto, Y. Awane, T. Kitayoshi, S. Fujiwara, S. Hashiguchi, Z. Terashita, M. Shiraishi and T. Watanabe, J. Cardiovasc. Pharmacol., 24, 929 (1994).
94. I . Cavero and J. Premmereur, Cardiovasc. Res., 24, 32 (1994).
95. G. Haeusler and I. Lues, Eur. Heart J., 15, 82 (1994).
96. M. Chiariello, C. Duilio and G. Ambrosio, Cardiologia., 38, 459 (1993).

97. K. Satoh, T. Mori, H. Yamada, and N. Taira, Cardiovascular Drugs and Therapy, 7, 691 (1993).
98. H. Purcell and K. Fox, Br. J. Clin. Pract., 47, 150 (1993).
99. G. J. Gross, D. C. Warltier and K. A. Lamping, Pharmacol. Commun., 2, 353 (1993).
100. J. A. Auchampach and G. J. Gross, Eur. Heart J., 14, 10 (1993).
101. J. A. Auchampach, M. Maruyama and G. J. Gross, Eur. Heart J., 15, 89 (1994).
102. Z. Yao and G. J. Gross, Am. J. Physiol., 267, H1888 (1994).
103. J. A. Auchampach and G. J. Gross, J. Cardiovasc. Pharmacol., 23, 554 (1994).
104. J. Pignac, J. Bourgouin and L. Dumont, Can. J. Physiol. Pharmacol., 72, 126 (1994).
105. S. Rohmann, H. Weygandt, P. Schelling, L. K. Soei, K-H. Becker, P. D. Verdouw, I. Lues and G. Haeusler, Cardiovasc. Res., 28, 858 (1994).
106. J. A. Auchampach, M. Maruyama, I. Cavero and G. J. Gross, J. Pharmacol. Exp. Ther., 259, 961 (1991).
107. K. S. Atwal, G. J. Grover, S. Z. Ahmed, F. N. Ferrara, T. W. Harper, K. S. Kim, P. G. Sleph, S. Dzwonczyk and A. D. Russell, J. Med. Chem., 36, 3971 (1993).
108. K. Atwal and F. N. Ferrara, Patent Application, EP 610553 (1994).
109. Z. Yao and G. J. Gross, Circulation, 89, 1769 (1994).
110. A. A. M. Wilde and M. J. Janse, Cardiovasc. Res., 28, 16 (1994).
111. C. F. Toombs, T. L. Moore and R. J. Shebuski, Pharmacology, 49, 96 (1994).
112. C. F. Toombs, N. R. Norman, V. E. Groppi, K. S. Lee, R. C. Gadwood and R. J. Shebuski, J. Pharmacol. Exp. Ther., 263, 1261 (1992).
113. N. R. Norman, C. F. Toombs, S. A. Khan, L. V. Buchanan, M. G. Cimini, J. K. Gibson, K. D. Meisheri and R. J. Shebuski, Pharmacology, 49, 86 (1994).
114. S. Matzno, M. Gohda, M. Eda, H. Ebisu, S. Uno, N. Ishida, N. Nakamura and K. Yamanouchi, J. Pharmacol. Exp. Ther., 271, 1666 (1994).
115. I. Sugo, S. Yoshida, K. Satoh, K. Kamei, J. Imagawa, M. Akima, H. Nabata, A. Hayasaka and N. Chiba, 67th Annual Meeting. of the Japanese Pharmacological Society, Kyoto, Japan, March, 21, Abstract P583(1994).
116. J. Morley, Potassium Channel Openers. In: Drugs and the Lung (1994), 383-96. Edited by C. P. Page, W. J. Metzger, Publisher: Raven, New York, N. Y. 1995.
117. D. R. Buckle, Pulm. Pharmacol., 6, 161 (1993).
118. R. Small, J. L. Berry, S. J. Cook, R. W. Foster, K. A. Green, and M. Murray, Lung Biol. Health Dis., 67, 137 (1993).
119. R. Small, J. Berry and R. Foster, Braz. J. Med. Biol. Res., 25, 983 (1992).
120. R. C. Small, J. L. Berry, J. F. Burka, S. J. Cook, R. W. Foster, K. A. Green and M. A. Murray, Clin. Exp. Allergy, 22, 11 (1992).
121. S-F. Fan, S. Wang and C. Y. Kao, J. Gen. Physiol., 102, 257 (1993).
122. S-F. Fan, S. Wang and C. Y. Kao, Biochem. Biophys. Res. Commun., 200, 1341 (1994).
123. D. R. Buckle, D. S. Eggleston, I. L. Pinto, D. G. Smith and J. M. Tedder, Bioorg. Med. Chem. Lett., 2, 1161 (1992).
124. J. R. S. Arch, N. E. Bowring and D. R. Buckle, Pulm. Pharmacol., 7, 121 (1994).
125. J. Imagawa, K. Kamei, S. Yoshida, I. Sugo, T. Koga and H. Nabata, J. Pharmacol. Exp. Ther., 269, 1 (1994).
126. J. Imagawa, S. Yoshida, T. Koga, K. Kamei and H. Nabata, Gen. Pharmacol., 24, 1505 (1993).
127. K. Tamura, Y. Suzuki, S. Yoshida and H. Nabata, J. Cardiovasc. Pharmacol., 23, 220 (1994).
128. N. Cook and I. Chapman, Cardiovasc. Drugs. Ther., 7, 555 (1993).
129. J. F. Burka, J. L. Berry, R. W. Foster, R. C. Small and A. J. Watt, Br. J. Pharmacol., 104, 263 (1991).
130. J. Ishikawa, M. Ichinose, N. Nakajima, T. Takahashi, H. Yamauchi, M. Miura and K. Shirato, Am. J. Respir. Crit. Care. Med., 150, 1379 (1994).
131. J. C. Clapham, N. E. Bowring, B. K. Trail, D. A. Fuller and D. M. Good, Pulm. Pharmacol., 6, 201 (1993).
132. G. Edwards, M. Henshaw, M. Miller and A. Weston, Br. J. Pharmacol., 102, 679 (1991).
133. A. D. Bonev and M. T. Nelson , Am. J. Physiol., 264, C1190 (1993).
134. S. Swami, P. Abrams, Curr. Opin. Urol. 4, 192 (1994).
135. S. A. Antane, J. A. Butera, T. M. Argentieri, W. Norton, D. M. Zebick, W. Spinelli, T. Bridal, A. Wojdan, G. Oshiro and J. F. Bagli, 207th ACS National Meeting, San Diego, CA, March 13-18, Abstract 227 (1994).
136. J. A. Butera and S. A. Antane, Patent , US 5225566, (1993).
137. D. Mattia, T. Nagao, M. A. Rogawski and M. Avoli, Neuropharmacology, 33, 1515 (1994).
138. Y. Chugh, N. Saha, A. Sankaranarayanan and P. Sharma, Pharmacol. Toxicol., 73, 1 (1993).
139. K. K. Vong, J. M. Evans, G. M. M. G. Nadler and R. N. Willette, Patent Application, WO 94/13292 (1995).
140. S. P. Welch and L. D. Dunlow, J. Pharmacol. Exp. Ther., 267, 390 (1993).
141. M. Narita, K. Takamori, N. Kawashima, M. Funada, J. Kamei, T. Suzuki, M. Misawa and H. Nagase, Psychopharmacology (Berlin), 113, 11 (1993).
142. R. Etcheberrigaray, E. Ito, K. Oka, B. Tofel-Grehl, G. E. Gibson and D. L. Alkon, Neurosci. Res. Commun., 13, S7 (1993).
143. S. Oleson, P. Moldt and O. Pedersen, Patent Application, WO 94/22807 (1994).
144. D. D. Denson, P. Duchatelle and D. C. Eaton, Brain Res., 638, 61 (1994).
145. C. S. Harmon, D. Lutz and J. Ducote, Skin Pharmacol., 6, 170 (1993).
146. B. Nilius and G. Droogmans, News Physiol. Sci., 9, 105 (1994).
147. Y. S. Lee, M. M. Sayeed and R. D. Wurster, Pharmacology, 49, 69 (1994).

Chapter 10. Progress in the Development of Endothelin Receptor Antagonists

Thomas F. Walsh
Merck Research Laboratories, Rahway, NJ 07065

Introduction - Identification of the uniquely potent vasoconstrictive peptides endothelin-1, 2, and 3 (ET-1, 2, 3) in the late 1980's (1, 2), has stimulated an intense effort to delineate the complex pharmacology of the ET system. ETs are produced upon appropriate stimulation by a variety of cells including endothelial, epithelial, mesangial, smooth muscle, glial and neuronal cells and while it is apparent that they exert their effects in nearly all tissues, the coronary, renal, and cerebral vasculatures are especially sensitive to these peptides. In normal physiological states they contribute to the paracrine/autocrine control of vascular smooth muscle tone and circulating levels of ETs are generally very low. Consequently observations of elevated ET levels in certain disease states has led a number of laboratories to search for effective pharmacological tools with which to assess the role of ETs in pathophysiology.

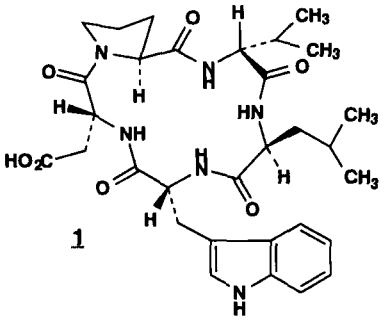
Extensive reviews of the biochemistry, pharmacology and physiology of the endothelins have been published (3, 4). The role of the endothelins in the control of cardiovascular and renal function, and their effects on the development of hypertension have also recently been examined (5-7). A current summary of the development of peptide agonists and antagonists of ET receptors is available and a review in *Annual Reports in Medicinal Chemistry* last year addressed progress in the development of small molecule ET antagonists (8, 9). A detailed perspective on the rationale for the development of ET antagonists also appeared last year, in addition to several other articles reviewing progress towards this goal (10-13). The first therapeutic agents based upon ET receptor blockade have recently begun clinical evaluation and considerable progress towards the identification of newer ET antagonists has been reported in the last year. Thus, highlights of recent progress in ET research and advances in the development of non-peptidic ET receptor antagonists will be the subject of this chapter.

Endothelin Receptors - ET receptors are differentiated by their relative affinities for the three peptide isoforms. Since ET-1 and ET-2 are rather similar agonists, a simplified classification emerges in which ET receptors are distinguished by the order of their affinities: ET-1 > ET-3, ET-1 = ET-3, and ET-3 > ET-1. Receptors displaying these patterns of affinities have been designated ET_A, ET_B and ET_C respectively, and this classification was recently endorsed by the International Union of Pharmacology (14). While pharmacological evidence for an ET_C receptor expressed in the dermal melanophores and heart of *Xenopus laevis* has accumulated (15, 16), it has not yet been possible to identify a mammalian counterpart and Southern blot analysis of human DNA has only revealed the genes for ET_A and ET_B subtypes (17).

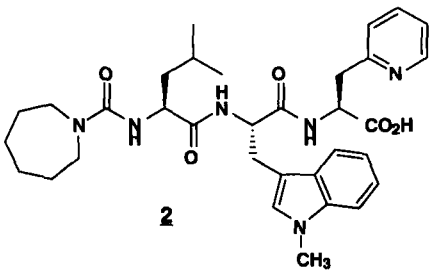
Debate continues concerning the role played by ET_B receptors in mediating ET-induced vasoconstriction in humans and a recent report of the cloning of two distinct human ET_B receptors by alternative splicing of a single gene further complicates this issue (18). The observations that ET-3 is much less potent than ET-1 in constriction of human vessels, that the ET_A-selective antagonist BQ-123 (1) blocks vasoconstriction in most human vessels, and that selective ET_B agonists such as BQ-3020 fail to induce potent vasoconstriction has led to the conclusion that vasoconstriction in man appears to be largely an ET_A mediated phenomenon (19). Yet recent experiments using the ET_A selective antagonists BQ-123 (1) and FR139317 (2), and the combined ET_A/ET_B non-peptidic antagonist bosentan (3) in human

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

mammary arteries and veins have provided compelling evidence that both receptor subtypes are involved in ET-induced vasoconstriction (20). The heterogeneity of ET receptor distribution in human coronary arteries, and the predominance of ET_B receptors in the human kidney further suggest that in at least some pathological states, blockade of both receptor subtypes may be essential to prevent the sequelae associated with elevated ET levels in man (21, 22).



1



2

Endothelin Physiology - Two recent reports have revealed that endothelin plays an important role during development. The ET-1 gene in mouse embryonic stem cells was disrupted to raise a strain of transgenic animals deficient in ET-1 (23). The $ET\text{-}1^{-/-}$ homozygous mice have abnormalities of the pharangeal arch and die of respiratory failure at birth. $ET\text{-}1^{-/+}$ heterozygous littermates survive but exhibit 40-50% lower plasma ET-1 levels than wild type mice. Paradoxically blood pressure at 8-10 weeks is mildly (11 mm Hg) elevated possibly because in physiological states ET-1 regulates blood pressure as a depressor agent. A congenital mutation of the ET_B receptor gene has been identified in patients with Hirschsprung's disease. This defect is characterized by an absence of enteric ganglia in the distal colon and was traced to a recessive susceptibility locus on chromosome 13, which was subsequently shown to be the ET_B receptor gene. A G→T missense mutation substitutes the highly conserved Trp-276 residue in transmembrane helix 5 with a Cys residue resulting in partial impairment of ligand-induced Ca^{2+} transient levels (24).

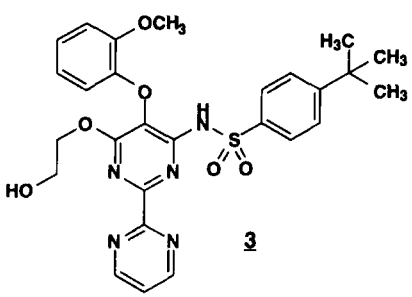
Electrophysiological studies of the actions of endothelin on ion channels in cardiac muscle provides evidence on the protective effects of ET in ischemia. ET-1 inhibits the protein kinase A-dependent chloride current in voltage-clamped guinea pig ventricular myocytes. This catecholamine-dependent current opposes the L-type calcium current by shortening the action potential duration, therefore ET-1 secreted by cardiac endothelial cells acts in a paracrine fashion to moderate the risk of ventricular arrhythmias that may occur in the course of a heart attack (25). A related study showed that ET-1, but not ET-3, inhibited the L-type calcium current in mammalian atrial monocytes, and that ET-1 strongly reduced the heart rate when it was increased by β-adrenoreceptor stimulation (26).

Endothelin Converting Enzyme - Biosynthesis of ET-1, 2, and 3 involves several post-translational sequence specific cleavages of prohormonal peptides that are encoded by separate genes. The final step in each cascade is the proteolytic cleavage of the 39 amino acid containing peptides big ET-1, 2 and 3 between Trp-21 and Val-22 residues by specific endothelin converting enzymes (ECEs). ECEs have long been recognized as attractive targets for pharmacological intervention in disease states associated with elevated ET levels, therefore numerous efforts to isolate and characterize the ECEs have been undertaken. Encouraging reports have appeared this year from several laboratories including the cloning and expression of human and bovine ECEs and methodology for the isolation of human ECE free from contamination by neutral endopeptidase-24-11 (27-31). Previous efforts to develop ECE inhibitors have been met with limited success, however if these reports of the cloning and characterization of ECEs have identified the physiologically relevant enzymes, then the prospects for the discovery of therapeutically useful ECE inhibitors may be significantly improved (11).

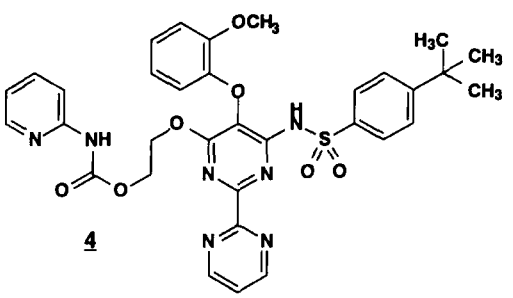
Structural Studies - The crystal structure of human endothelin has been determined to 2.18 Å resolution (32). Examination of the structure reveals that the carboxy-terminal region is helical and thus differs significantly from the various models derived from NMR data. The biological relevance of the structure was justified with arguments that the crystallization was conducted in water while NMR studies have been carried out in organic solvent/water mixtures, that the distance between residues Tyr-13 and Trp-21 in the tail is 14-15 Å which is in good agreement with fluorescence energy transfer experiments, and finally that the structure correlates well with the physiological properties of mutant analogs.

Endothelin Antagonists - Early efforts undertaken by several laboratories to develop ET antagonists focused upon synthetic modification of the ET agonist peptides as well as classical SAR studies conducted with natural product screening leads. These strategies identified a number of highly potent peptidic antagonists such as PD 142893, PD 145065, and BQ-123 (1) among others, and these agents have been capably discussed in the reviews cited above (8-13). Although these compounds may not advance into clinical development due to limiting pharmacokinetics, they are nonetheless useful pharmacological tools which continue to be widely employed in ET research. Especially broad availability of the ET_A-selective cyclic pentapeptide BQ-123 has led to its investigation in numerous disease models and has helped to establish the utility of ET antagonists for some of these indications. In some studies reported this year, BQ-123 was shown to be beneficial in ischemia-induced (33), cyclosporine-induced (34), and transplantation-associated (35) renal failure, hypertension (36), and stroke (37). Infusion of BQ-123 into human brachial arteries was shown to effect vasodilatation attended by blood flow increases of 64% after an hour, demonstrating a role for endogenous ET-1 in the maintenance of basal vascular tone in man (38).

Pharmacological evaluation of the sulfonamidopyrimidine based ET antagonist Ro 46-2005 which was reviewed here last year (9) has been extended (39-42), and detailed characterization of bosentan (43), a more potent antagonist from this series has now published. Bosentan (Laroteline®, Ro 47-0203, 3), competitively antagonizes the specific binding of [^{125}I]ET-1 to human smooth muscle cells (ET_A) with a K_i value of 4.7 nM and to human placenta membranes (90% ET_B) with a K_i of 95 nM (44). Functionally, bosentan inhibits the ET-1 induced contraction of rat aorta and the sarafotoxin S6c induced contraction of rat trachea with pA_2 values of 7.2 and 6.0 respectively, and it is a selective, orally active antagonist. Binding experiments using chimeric ET receptors demonstrated that both ET-1 and bosentan interact with the (140-144) KLLAG sequence located at the carboxyl terminus of the second transmembrane region of the ET_A receptor (45). The ability of bosentan to block hemodynamic responses to exogenous ET and big ET-1 challenges in conscious rats was reported (46). Since bosentan had no influence on the recovery of cardiac function during reperfusion following ischemia, it was suggested that endogenous ET does not contribute to coronary endothelial injury in this model (47, 48). However administration of bosentan to rats with chronic heart failure (CHF) was shown to lower mean arterial pressure (49). Furthermore, recently reported data from a clinical trial of



3

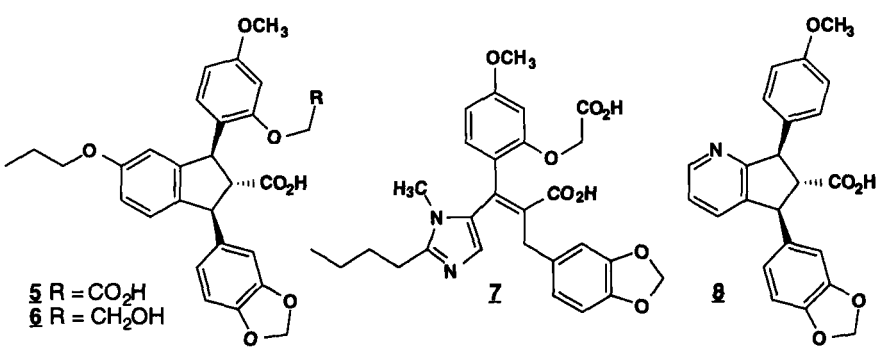


4

bosentan in patients with severe CHF has confirmed this benefit in man. Patients administered bosentan (100 mg followed after 60 min by 200 mg i.v.) exhibited sustained systemic and venous vasodilatation and improved cardiac performance without reflex tachycardia (50). Additional studies have shown that bosentan attenuates ET-1 induced reduction of feline pial arteriole caliber providing a role for ET antagonists in determining the functional significance of ETs in the cerebrovasculature (51). Bosentan provides protection from ET-1 induced changes in gastric mucosal hemodynamics and the development of ET-1 induced gastric ulcer in rats, and finally that it inhibits the ET-1 induced vasoconstriction in rat skin vasculature (52, 53).

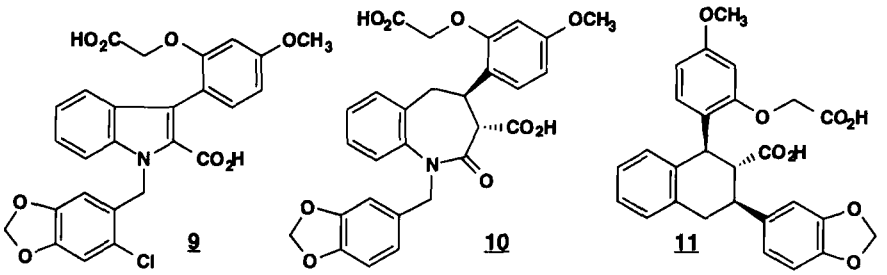
A recently published patent application which claims urethanes of the sulfonamido-pyrimidine based ET antagonists provides the first report of new analogs which may have considerably improved potency (54). The bosentan analog **4** has an IC_{50} of 6 nM for binding to cloned human ET_A receptors, a $pA_2 = 9.0$ for the blockade of the ET-1 induced contractions in rat aortic rings, and in "stroke prone" spontaneously hypertensive rats a 30% reduction in mean arterial pressure (MAP) after oral administration (30 mg/kg) was reported.

The discovery of the indane-2-carboxylic acid non-selective ET antagonist SB 209670 (**5**) was also noted last year and a considerable body of new information concerning this antagonist has appeared in the interim (8). A communication has described its development from a screening lead that was synthetically modified using peptidomimetic arguments arising from inspection of modeling overlays with an NMR-derived conformation of ET-1 (55). SB 209670 potently inhibits the binding of [^{125}I]ET-1 to cloned human ET receptors with K_i values of 0.43 ± 0.09 and 14.7 ± 3.0 nM for ET_A and ET_B subtypes, and is a competitive, functional antagonist (56). The dextrorotatory enantiomer [(+)-(1*S*, 2*R*, 3*S*) as shown] is the more potent antipode, and is significantly more potent than the peptidic antagonists BQ-123 (**1**), PD 142,893, RES-701-1, or the non-peptide bosentan (**3**) (57). SB 209670 concentration-dependently blocks ET-1 induced vasoconstriction in vitro (56) and in vivo (58), dose-dependently lowers blood pressure in spontaneously hypertensive rats, and protects against ischemia-induced neuronal degeneration in a gerbil stroke model (59). ET-1 levels are elevated in the coronary sinus following percutaneous transluminal coronary angioplasty (PTCA) (60). Since vascular restenosis frequently limits the long term efficacy of this procedure and ET-1 is a potent mitogen, SB 209670 was evaluated in a rat model of neointimal formation following PTCA. SB 209670 blocked ET-1 induced [^{3}H]thymidine incorporation in rat aortic vascular smooth muscle cells in vitro and was partially efficacious (50%) in reducing exogenous ET-1 induced neointimal formation following PTCA in rats suggesting that ET antagonists may be a useful adjunct in these procedures (61). Evidence has accumulated that ET plays an important role in the



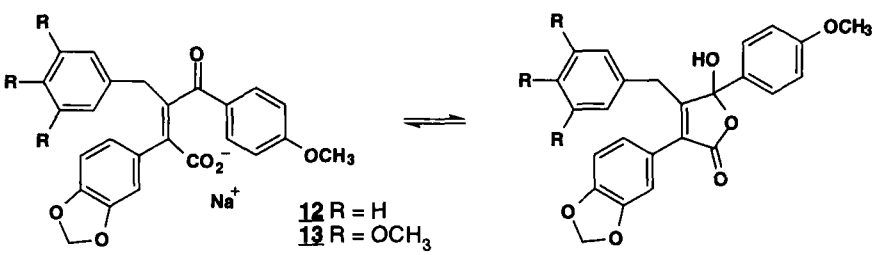
pathogenesis of acute renal failure (ARF) and SB 209670 was shown to be beneficial in ischemia-induced ARF in both rats and dogs, possibly by improving tubular reabsorption of Na^+ and moderately increasing glomerular filtration rate and K^+ excretion (33, 62, 63).

SB 209670 (5) has modest oral bioavailability and is reported to be in clinical evaluation as an intravenous formulation for the prophylaxis/treatment of radiocontrast dye-induced ARF (64). A close analog of 5 (SB 217242, 6) having an hydroxyethoxy side chain in place of the oxyacetic acid has recently been described (65, 66). Although SB 217242 is somewhat less potent than SB 209670 displaying apparent K_i values of 1.1 nM for ET_A and 110 nM for ET_B cloned human receptors, it has significantly improved oral bioavailability (60% in rats) and is being developed as an oral formulation for other potential ET mediated indications (64).



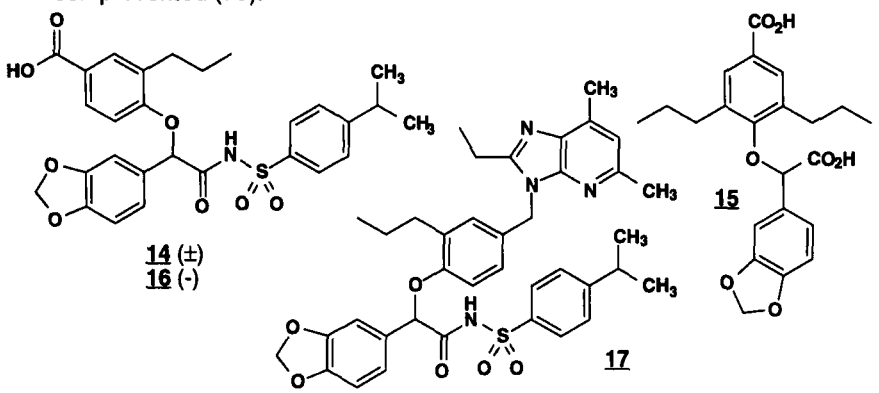
A number of new designs for ET antagonists structurally related to the indane-2-carboxylic acids have recently appeared in the patent literature including arylpropenoic acid derivatives such as SB 209834 (7) (ET_A = 4 nM; ET_B = 500 nM) (67), heteroaromatic ring-fused cyclopentene derivatives such as BQ-4269 (8) (68), indole derivatives (9) (69), cyclopentane derivatives (70), benzazepine-2-ones (10) (71), and naphthalene-2-carboxylic acids (11) (72). Details of the pharmacological properties of compounds from these newer series have not yet appeared.

A new series of ET_A-selective antagonists has been developed from a weakly active butenolide lead discovered through screening (73). Optimization of the potency of this series utilizing a Topliss operational scheme for analog synthesis afforded the ET_A-selective antagonists PD 155080 (12) and PD 156707 (13) (74). PD 155080 competitively antagonizes [^{125}I]ET-1 binding to cloned human ET_A receptors expressed in Ltk- cells with an IC_{50} value of 7.8 nM and blocks [^{125}I]ET-3 binding to CHO expressed human ET_B receptors with an IC_{50} value of 3.5 μM. PD 156707 is approximately ten fold more potent having IC_{50} values of 0.3 nM and 0.42 μM in these ET_A and ET_B receptor cell lines. In a functional assay, PD 156707 antagonized ET-1 induced arachidonic acid release from rabbit renal artery vascular smooth muscle cells with an IC_{50} of 1.1 nM compared to 0.15 μM for PD 155080. These compounds undergo a unique pH-dependent tautomerism between closed hydroxybutenolide and ring opened ketoacid forms which may account for their reported ability to penetrate the blood brain barrier (74, 75). The soluble salt forms of PD 155080 and PD 156707 are both orally bioavailable in rat (87% and 41%) and have terminal elimination $t_{1/2}$ values of approximately 5 and 1 hour respectively. The ability of these compounds to



penetrate the CNS and their profile as ET_A-selective agents suggests that they may be especially appropriate for blocking the deleterious effects of the endothelins associated with cerebral vasospasm and subarachnoid hemorrhage.

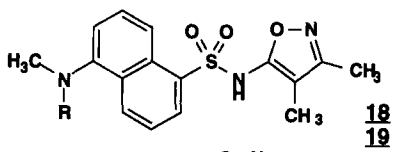
Several pharmaceutical companies have noted the discovery of ET antagonist leads through screening collections of angiotensin II (AII) antagonists. One laboratory recently described identification of weak potency within a series of α-phenoxyphenylacetic acid based AII antagonists and subsequent SAR studies which led to the potent, and selective acylsulfonamide derivative L-749,329 (**14**) (76). L-749,329 was shown to antagonize the specific binding of [^{125}I]ET-1 in a previously described cloned human receptor CHO cell line with K_i values of 0.13 and 5.4 nM on ET_A and ET_B respectively (77). L-749,329 competitively antagonizes ET-1 induced contractions in rabbit iliac artery rings with a pA_2 value of 7.7, and in conscious rats it blocks ET-1 induced pressor responses with an AD_{50} = 7.3 mg/kg (i.v.) with a duration of action greater than 2 hours. Following oral administration, L-749,329 is 60% bioavailable in rats and 49% in dogs. A related dicarboxylic acid series exemplified by L-747,844 (**15**) was also described which had IC_{50} values of 48 and 630 nM on ET_A and ET_B receptors respectively and is 26% orally bioavailable in the rat (78). Racemic L-749,329 was resolved and details of the pharmacological characterization of the single active enantiomer (-)-L-754,142 (**16**) (K_i's: ET_A = 0.062 nM; ET_B = 2.25 nM) have been presented (79).



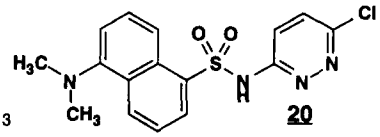
A report of the initial synthetic modifications of the α-phenoxyphenylacetic acid screening leads was made and the identification of a novel dual AII/ET antagonist (IC_{50}s: ET_A = 24 nM, ET_B = 60 nM, AT_1 = 11 nM, AT_2 = 47 nM) L-747,072 (**17**) was noted (80). The discovery of modestly active compounds with the ability to block both AII and ET receptors suggests the possibility that it may be possible to advance a single agent for the management of hypertension associated with elevated levels of both of these hormones. Indeed, another company has recently published a patent application claiming pharmaceutical combinations of renin-angiotensin (RAS) inhibitors with ET antagonists for the treatment of hypertension (81). Efficacy was demonstrated in "stroke-prone" spontaneously hypertensive rats using combinations of bosentan (**3**) with the ACE inhibitor cilazapril as well as in sodium-depleted squirrel monkeys with bosentan and the renin inhibitor remikiren. In both experiments the combination therapies were shown to decrease mean arterial pressure below levels that could be achieved with either RAS blocker alone.

The ET_A selective naphthalenesulfonamide BMS-182874 (**18**) is orally active with greater than 24 hours duration of action for lowering MAP in DOCA-salt hypertensive rats (82). Details of the structure-activity relationships for this series of compounds recently published (83). The long duration of action observed for BMS-182874 was reported to derive in part from a relatively rapid *N*-demethylation in vivo which affords a biologically active metabolite (**19**) that is only slowly cleared from rat plasma (84). Conformational molecular field analysis for 36 sulfonamides in this series enabled discrimination between two hypothetical biologically active conformations and these superpositions were shown to be consistent with receptor-modeling and mutagenesis experiments showing that Tyr-129 plays an important role in binding this series of ligands (85, 86). A patent application claiming related *N*-pyridazinyl

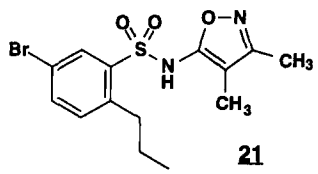
substituted sulfonamides such as (**20**) has recently appeared from the same group (87).



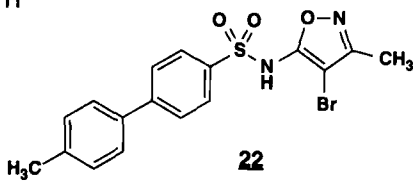
18 R = CH_3
19 R = H



20



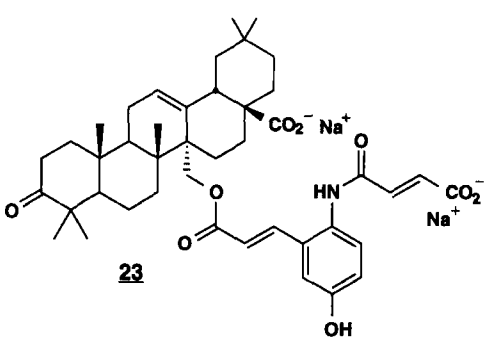
21



22

A second group has investigated isoxazolylsulfonamide derivatives and reported structural features influencing ET_A or ET_B-selectivity for these antagonists. The 2,5-disubstituted benzenesulfonamides such as (**21**) are ET_A selective (IC_{50} = 15 nM), while the biphenyl derived series favors ET_B activity. IPI-950 (**22**) is 290 fold selective, exhibiting an IC_{50} of 17 nM for binding to human ET_B receptors expressed in COS7 cells, but had an IC_{50} of 2.0 μM in a functional assay of ET-1-stimulated phosphatidyl inositol hydrolysis. A 4-halo substituent was reported to enhance potency in both series (88-90).

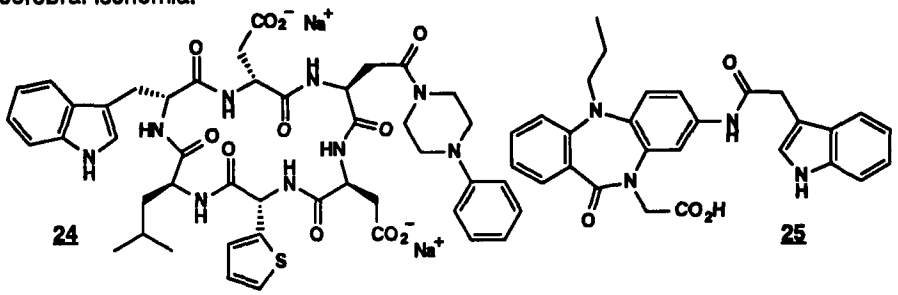
The ET_A-selective antagonists FR139317 (**2**) and 97-139 (**23**) continue to elicit considerable interest. The inability of FR139317 to completely inhibit ET-induced vasoconstriction in rat kidney (91) and in guinea-pig airways (92) demonstrated a role for ET_B-like receptors in these tissues. FR139317 attenuated ET-1-induced vasoconstrictor effects but not infarct size in a rabbit model of myocardial-ischemia and reperfusion (93), however pretreatment by **22** with a 15 mg/kg i.v. infusion provided significant protection (20%) in a rat myocardial-infarction model (94). Synthetic modification of a natural product isolated from the bayberry *Myrica cerifera* (50-235) afforded 97-139 (**23**). Pharmacological studies indicate that 97-139 displaces the specific binding of [^{125}I]ET-1 to rat aortic smooth muscle A7r5 cells (ET_A) and to Girardi heart cells (ET_B) with K_i values of 1.0 ± 0.2 and 1000 ± 200 nM respectively, but there is no functional inhibition of ET_B receptors (95). 97-139 is reported to have equivalent in vivo potency to BQ-123 but has a large (22-24 fold) shift in potency in the presence of 95% plasma and 4% albumin.



23

Characterization of the new cyclic hexapeptide antagonist TAK-044 (**24**) has been reported. TAK-044 has high affinity for ET_A receptors having an IC_{50} = 0.082 nM (ET_B = 120 nM), and was shown to produce long lasting inhibition of ET-mediated blood pressure responses in anesthetized rats (96-98). Administration of **24** at 10 mg/kg i.v. nearly completely inhibited the ET-1 (0.3 nmol/kg i.v.) induced pressor response. In contrast to the ET_A-selective cyclic pentapeptide BQ-123, TAK-044 (1 and 10 mg/kg) completely blocks the ET_B mediated transient depressor response induced by ET-1 as well as the depressor and pressor responses induced by the ET_B selective agonist sarafotoxin S6c. TAK-044 competitively inhibits [^{125}I]ET-1 binding to rat renal membranes with an IC_{50} of 0.34 nM and in a rat renal ischemia model of acute renal failure it dose-dependently (1-10 mg/kg i.v.) partially attenuated increases in plasma

creatinine and morphological damages of the kidney (99). TAK-044 was reported to have recently entered Phase I trials for myocardial infarction, acute renal failure and cerebral ischemia.



24 25

Molecular modeling studies of BQ-123 led to the proposal that certain rigid tricyclic templates might mimic the peptide scaffold. Synthesis of a series of dibenzodiazepine-10-acetic (25) acids having structural features suggested by overlays with BQ-123 (1) provided several analogs with micromolar affinities for ET_A and ET_B receptors (100).

Conclusions - The recent availability of a variety of potent and selective endothelin receptor antagonists provides the opportunity to elucidate further the role of endothelin in physiology as well as in pathophysiological states. The rapid progress in the identification of ET_A-selective and ET_A/ET_B balanced antagonists suggests that the introduction of superior ET_B-selective compounds and possibly ECE inhibitors can be anticipated in the near future. As experience with this new class of therapeutic agents accumulates, it should also be possible to define which of the numerous potential disease indications are the most appropriate targets for ET blockade.

References

1. M. Yanagisawa, H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, T. Masaki, Nature, 332, 411 (1988).
2. A. Inoue, M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, K. Goto, T. Masaki, Proc. Natl. Acad. Sci. USA, 86, 2863 (1989).
3. J.P. Huggins, J.T. Pelton, R.C. Miller, Pharmacol. Ther., 59, 55 (1993).
4. G.M. Rubanyi, M.A. Polokoff, Pharmacol. Reviews, 46, 325 (1994).
5. G. Remuzzi, A. Benigni, The Lancet, 342, 589 (1993).
6. P.M. Vanhoutte, Hypertension, 21, 747 (1993).
7. B.K. Krämer, M. Ackermann, S.M. Kohler, G.A.J. Riegger, Clin. Invest., 72, 88 (1994).
8. W.L. Cody and A.M. Doherty, Biopoymers, 37, 89 (1995).
9. D.C. Spellmeyer, Annu. Rep. Med. Chem., 29, 65 (1994).
10. T.D. Warner, B. Battistini, A.M. Doherty, R. Corder, Biochem. Pharmacol., 48, 625 (1994).
11. X.M. Cheng, S.S. Nikam, A.M. Doherty, Curr. Med. Chem., 1, 271 (1994).
12. C. Wilson, R.B. Hargreaves, Progress in Medicinal Chemistry; G.P. Ellis, D.K. Luscombe, Eds.; Elsevier Science, The Netherlands, 31, pp. 371-410 (1994).
13. S. Moreland, Can. J. Physiol. Pharmacol., 72, 1469 (1994).
14. T. Masaki, J.R. Vane, P.M. Vanhoutte, Pharmacol. Reviews, 46, 137 (1994).
15. S. Kame, C.K. Jayawickreme, M.R. Lerner, J. Biol. Chem., 268, 19126 (1993).
16. C. Kumar, V. Mwangi, P. Nuthulaganti, H.-L. Wu, M. Pullen, K. Brun, H. Aiyar, R.A. Morris, R. Naughton, P. Nambi, J. Biol. Chem., 269, 13414 (1994).
17. A. Sakamoto, M. Yanagisawa, T. Sakurai, Y. Tukuwa, H. Yanagisawa, T. Masaki, Biochem. Biophys. Res. Commun., 178, 656 (1991).
18. V. Shyamala, T.H.M. Moulthrop, J. Stratton-Thomas, P. Tekamp-Olson, Cell. Molec. Biol. Res., 40, 285 (1994).
19. A. Davenport, J.J. Maguire, Trends Pharmacol. Sci., 15, 9 (1994).
20. B. Seo, B.S. Oemar, R. Siebenmann, L. von Segesser, T.F. Lüscher, Circulation, 89, 1203 (1994).
21. T. Godfraind, Br. J. Pharmacol., 110, 1201 (1993).
22. F.E. Karet, R.E. Kuc, A.P. Davenport, Kidney Int., 44, 36 (1993).
23. Y. Kurihara, H. Kurihara, H. Suzuki, T. Kodama, K. Maemura, R. Nagai, H. Oda, T. Kuwaki, W.-H. Cao, N. Kamada, K. Jishage, Y. Ouchi, S. Azuma, Y. Toyoda, T. Ishikawa, M. Kumada, Y. Yazaki, Nature, 368, 703 (1994).

24. E.G. Puffenberger, K. Hosada, S.S. Washington, K. Nakao, D. deWit, M. Yanagisawa, A. Chakravarti, Cell, 79, 1257 (1994).
25. A.F. James, L.-H. Xie, Y. Fujitani, S. Hayashi, M. Horie, Nature, 370, 297 (1994).
26. K. Ono, G. Tsujimoto, A. Sakamoto, K. Eto, T. Masaki, Y. Ozaki, M. Satake, Nature, 370, 301 (1994).
27. D. Xu, N. Emoto, A. Giaid, C. Slaughter, S. Kaw, D. deWit, M. Yanagisawa, Cell, 78, 473 (1994).
28. T. Ikura, T. Sawamura, T. Shiraki, H. Hosokawa, T. Kido, H. Hoshikawa, K. Shimada, K. Tanzawa, S. Kobayashi, S. Miwa, T. Masaki, Biochem. Biophys. Res. Commun., 203, 1417 (1994).
29. M. Schmidt, B. Kroger, E. Jacob, H. Seulberger, T. Subkowski, R. Otter, T. Meyer, G. Schmalzing, H. Hillen, FEBS Letters, 356, 238 (1994).
30. K. Shimada, Y. Matsushita, K. Wakabayashi, M. Takahashi, A. Matsubara, Y. Iijima, K. Tanzawa, Biochm. Biophys. Res. Commun., 207, 807 (1995).
31. R. Corder, N. Khan, V.J. Harrison, Biochem. Biophys. Res. Commun., 207, 355 (1995).
32. R.W. Janes, D.H. Peapus, B.A. Wallace, Nature, Struct. Biol., 1, 311 (1994).
33. M. Gellai, M. Jugus, T. Fletcher, R. DeWolf, P. Nambi, J. Clin. Invest., 93, 900 (1994).
34. S.D. Kivlighn, R.A. Gabel, P.K.S. Siegl, Kidney Int., 45, 131 (1994).
35. Y. Yokoi, S. Nakamura, A. Serizawa, R. Nishiyama, Y. Nishiwaki, S. Baba, Transplantation, 58, 144 (1994).
36. S.A. Douglas, M. Gellai, M. Ezekiel, E.H. Ohlstein, J. Hypertens., 12, 561 (1994).
37. G. Feuerstein, J.L. Gu, E.H. Ohlstein, F.C. Barone, T.L. Yue, Peptides, 15, 467 (1994).
38. W.G. Haynes and D.J. Webb, The Lancet, 344, 852 (1994).
39. M. Clozel, V. Breu, G.A. Gray, B.M. Löffler, J. Cardiovasc. Pharmacol., 22, S377 (1993).
40. J.R. Wu-Wong, W.J. Chiou, K.E. Naugles Jr., T.J. Opgenorth, Life Sci., 54, 1727 (1994).
41. J.R. Wu-Wong, W.J. Chiou, Z.J. Huang, M.J. Vidal, T.J. Opgenorth, Am. J. Physiol., 267, C1185 (1994).
42. B. Battistini, T.D. Warner, A. Fournier, J.R. Vane, Eur. J. Pharmacol., 252, 341 (1994).
43. S.P. Roux, M. Clozel, U. Sprecher, G. Gray, J.P. Clozel, Circulation, 88, I-170 (1993).
44. M. Clozel, V. Breu, G.A. Grey, B. Kalina, B.-M. Löffler, K. Burri, J.-M. Cassal, G. Hirth, M. Müller, W. Neidhart, H. Ramuz, J. Pharmacol. Exp. Ther., 270, 228 (1994).
45. M. Adachi, Y. Furuichi, C. Miyamoto, Eur. J. Pharmacol. Molec. Pharmacol., 269, 225 (1994).
46. S.M. Gardner, P.A. Kemp, J.E. March, T. Bennett, Br. J. Pharmacol., 112, 823 (1994).
47. P.H. Dagassan, V. Breu, M. Clozel, J.-P. Clozel, J. Cardiovasc. Pharm., 24, 867 (1994).
48. V. Richard, N. Kaeffer, M. Hogie, C. Tron, T. Blanc, C. Thuillez, Br. J. Pharmacol., 113, 869 (1994).
49. J.R. Teerlink, B.-M. Löffler, P. Hess, J.-P. Maire, M. Clozel, J.-P. Clozel, Circulation, 90, 2510 (1994).
50. W. Kiowski, O. Bertel, G. Sütsch, P. Hunziker, P. Müller, R. Schmitt, J. Kim, E. Oechslin, J. Am. College Cardiol., Special Issue, p. 296A, (1995).
51. T.R. Patel, M.A. McAuley, J. McCulloch, Eur. J. Pharmacol., 260, 65 (1994).
52. S. Lazaratos, A. Nakahara, K. Goto, H. Fukutomi, Life Sci., 56, PL-195 (1995).
53. E. Lawrence, S.D. Brain, Eur. J. Pharmacol., 260, 103 (1994).
54. V. Breu, K. Burri, J.-M. Cassal, M. Clozel, G. Hirth, B.-M. Löffler, M. Müller, W. Neidhart, H. Ramuz, EP 0,633,259-A1 (1995).
55. J.D. Elliott, M.A. Lago, R.D. Cousins, A. Gao, J.D. Leber, K.F. Erhard, P. Nambi, N.A. Elshourbagy, C. Kumar, J.A. Lee, J.W. Bean, C.W. DeBrosse, D.S. Eggleston, D.P. Brooks, G. Feuerstein, R.R. Ruffolo, Jr., J. Weinstock, J.G. Gleason, C.E. Peishoff, E.H. Ohlstein, J. Med. Chem., 37, 1553 (1994).
56. P. Nambi, N. Elshourbagy, H.-L. Wu, M. Pullen, E.H. Ohlstein, D.P. Brooks, M.A. Lago, J.D. Elliott, J.G. Gleason, R.R. Ruffolo, Jr., J. Pharmacol. Exp. Ther., 271, 755 (1994).
57. E.H. Ohlstein, G.R. Beck, Jr., S.A. Douglas, P. Nambi, M.A. Lago, J.G. Gleason, R.R. Ruffolo, Jr., G. Feuerstein, J.D. Elliott, J. Pharmacol. Exp. Ther., 271, 762 (1994).
58. S.A. Douglas, R.M. Edwards, J.D. Elliott, E.H. Ohlstein, Br. J. Pharmacol., 114, 405 (1995).
59. E.H. Ohlstein, P. Nambi, S.A. Douglas, R.M. Edwards, M. Gellai, A. Lago, J.D. Leber, R.D. Cousins, A. Gao, J.S. Frazee, C.E. Peishoff, J.W. Bean, D.S. Eggleston, N.A. Elshourbagy, C. Kumar, J.A. Lee, T.-L. Yue, C. Louden, D.P. Brooks, J. Weinstock, G. Feuerstein, G. Poste, R.R. Ruffolo, Jr., J.G. Gleason, J.D. Elliott Proc. Natl. Acad. Sci. USA, 91, 8052 (1994).
60. A. Tahara, M. Kohno, S. Yanagi, H. Itagane, I. Toda, K. Akioka, M. Teragaki, M. Yasuda, K. Takeuchi, T. Takeda, Metabolism, 40, 1235 (1992).
61. S.A. Douglas, C. Louden, L.M. Vickery-Clark, B.L. Storer, T. Hart, G.Z. Feuerstein, J.D. Elliott, E.H. Ohlstein, Circ. Res., 75, 190 (1994).
62. M. Gellai, M. Jugus, T.A. Fletcher, P. Nambi, D.P. Brooks, E.H. Ohlstein, J.D. Elliott, J. Gleason, R.R. Ruffolo, Jr. Fed. Am. Soc. Exp. Biol. J., 8, A260 (1994).
63. D.P. Brooks, D. DePalma, M. Gellai, P. Nambi, E.H. Ohlstein, J.D. Elliott, J.G. Gleason, R.R. Ruffolo, Jr., J. Pharmacol. Exp. Ther., 271, 769 (1994).
64. Scrip No. 1992, page 12 (January 20, 1995).
65. R.D. Cousins, J.D. Elliott, M.A. Lago, J.D. Leber, C.E. Peishoff WO 94/25013-A1, (1994).
66. J.D. Elliott, M.A. Lago, R.D. Cousins, A. Gao, J.D. Leber, J.A. Lee, P. Nambi, J. Weinstock, J.G. Gleason, C.E. Peishoff, E.H. Ohlstein, XIIIth International Symposium on Medicinal Chemistry, Paris, France, Sept. 1994, Abstract O35.
67. D.L. Bryan, J.D. Elliott, WO 94/02474-A1 (1994).

68. K. Ishikawa, T. Nagase, T. Mase, T. Hayama, M. Ihara, M. Nishikibe, M. Yano, U.S. Patent 5,389,620 (1995).
69. J.D. Elliott, J.D. Leber, WO 94/14434-A1 (1994).
70. J.D. Elliott, D. Bryan, WO 94/24084-A1 (1994).
71. J.D. Elliott, J.G. Gleason, D.T. Hill, WO 95/04534-A1 (1995).
72. J.D. Elliott, WO 95/04530-A1 (1995).
73. K.A. Berryman, A.M. Doherty, J.J. Edmunds, W.C. Patt, M.S. Plummer, J.T. Repine, WO 95/05376-A1 (1995).
74. A.M. Doherty, W.C. Patt, J.J. Edmunds, K.A. Berryman, B.R. Reisdorph, M.S. Plummer, A. Shahripour, C. Lee, X.-M. Cheng, D.M. Walker, S.J. Haleen, J.A. Keiser, M.A. Flynn, K.M. Welch, H. Hallak, D.G. Taylor, E.E. Reynolds, J. Med. Chem., 38, 1259 (1995).
75. T.R. Patel, S. Galbraith, M.A. McAuley, A.M. Doherty, J. McCulloch, Fourth International Conference on Endothelin, London, UK, Apr. 1995, Abstract C63.
76. T.F. Walsh, K.J. Fitch, P.K. Chakravarty, D.L. Williams, Jr., K.A. Murphy, N.A. Nolan, J.A. O'Brien, E.V. Lis, Jr., D.J. Pettibone, S.D. Kivlighn, R.A. Gabel, G.J. Zingaro, S.M. Krause, P.K.S. Siegl, B.V. Clineschmidt, W.J. Greenlee, 208th American Chemical Society National Meeting, Washington, D.C., Aug. 1994, Abstract MEDI 145.
77. D.L. Williams, Jr., K.L. Jones, K. Alves, C.P. Chan, G.F. Hollis, J.-S. Tung, Life Sci., 53, 407 (1993).
78. P.K. Chakravarty, D.S. Dhanoa, S. Bagley, J.R. Tata, W.J. Greenlee, D.L. Williams, Jr., B.V. Clineschmidt, D.J. Pettibone, XIIIth International Symposium on Medicinal Chemistry, Paris, France, Sept. 1994, Abstract O31.
79. D.L. Williams, Jr., K.L. Murphy, N.A. Nolan, J.A. O'Brien, D.J. Pettibone, S.D. Kivlighn, S.M. Krause, E.V. Lis, Jr., G.J. Zingaro, R.A Gabel, F.C. Clayton, P.K.S. Siegl, K. Zhang, J. Naue, K. Vyas, T.F. Walsh, K.J. Fitch, P.K. Chakravarty, W.J. Greenlee, B.V. Clineschmidt, J. Pharmacol. Exp. Ther., in press (1995).
80. T.F. Walsh, K.J. Fitch, D.L. Williams, Jr., K.L. Murphy, N.A. Nolan, D.J. Pettibone, R.S.L. Chang, S.S. O'Malley, B.V. Clineschmidt, D.F. Veber, W.J. Greenlee, Bioorg. Med. Chem Lett., 5, 1155 (1995).
81. J.-P. Clozel, M. Clozel, W. Osterrieder, EP 0,634,175-A1 (1995).
82. P.D. Stein, J.T. Hunt, D.M. Floyd, S. Moreland, K.E.J. Dickinson, C. Mitchell, E.C.-K. Liu, M.L. Webb, N. Murugesan, J. Dickey, D. McMullen, R. Zhang, V.G. Lee, R. Serafino, C. Delaney, T.R. Schaeffer, M. Kozlowski, J. Med. Chem., 37, 329 (1994).
83. P.D. Stein, D.M. Floyd, S. Bisaha, J. Dickey, R.N. Girotra, J.Z. Gougoutas, M. Kozlowski, V.G. Lee, E. C.-K. Liu, M.F. Malley, D. McMullen, C. Mitchell, S. Moreland, N. Murugesan, R. Serafino, M.L. Webb, R. Zhang, J.T. Hunt, J. Med. Chem. 38, 1344 (1995).
84. P.D. Stein, "Development of the Orally Active Sufonamide ET_A Antagonist BMS-182874" Endothelin Inhibitors: Advances in Therapeutic Application & Development, Philadelphia, PA, June 9-10, 1994.
85. S.R. Krystek, Jr., J.T. Hunt, P.D. Stein, T.R. Stouch, J. Med. Chem., 38, 659 (1995).
86. S.R. Krystek, Jr., P.S. Patel, P.M. Rose, S.M. Fisher, B.K. Kienzle, D.A. Lach, E.C.-K. Liu, J.S. Lynch, J. Novotny, M.L. Webb, J. Biol. Chem., 269, 12383 (1994).
87. J.T. Hunt, EP 0,640,596-A1 (1995).
88. M.F. Chan, "The Design, Synthesis and Biological Activity of Potent, Subtype-Selective Endothelin Antagonists" Endothelin Inhibitors: Advances in Therapeutic Application & Development, Philadelphia, PA, June 9-10, 1994.
89. M.F. Chan, B.G. Raju, A. Kois, E.J. Verner, C. Wu, R.S. Castillo, V. Yalamoori, V.N. Balaji, K. Ramnarayan, WO 94/27979-A1(1994).
90. M.F. Chan, B. Raju, A. Kois, R.S. Castillo, E.J. Verner, C. Wu, Y.V. Venkatachalapathi, I. Okun, E. Hwang, F.D. Stavros, V.N. Balaji, 209th American Chemical Society National Meeting, Anaheim, CA, April 2-6, 1995, Abstracts MEDI 167 & Medi 168.
91. R.P. Wellings, R. Corder, T.D. Warner, J.P. Cristol, C. Thiemermann, J.R. Vane, Br. J. Pharmacol., 111, 515 (1994).
92. B. Battistini, T.D. Warner, A. Fournier, J.R. Vane, Br. J. Pharmacol., 111, 1009 (1994).
93. L. McMurdo, C. Thiemermann, J.R. Vane, Br. J. Pharmacol., 112, 75 (1994).
94. J.Y. Lee, R.B. Warner, A.L. Adler, T.J. Opgenorth, Pharmacology, 49, 319 (1994).
95. S.-I. Mihara, S. Nakajima, S. Matumura, T. Kohnoike, M. Fujimoto, J. Pharmacol. Exp. Ther., 268, 1122 (1994).
96. Y. Awane, K. Kusumoto, K. Kubo, A. Kawata, T. Kikuchi, M. Wakimasu, T. Watanabe, M. Fujino, Jpn. J. Pharmacol., 64: Suppl I,167 P, (1994).
97. T. Kikuchi, T. Ohtaki, A. Kawata, T. Imada, T. Asami, Y. Masuda, T. Sugo, K. Kusumoto, K. Kubo, T. Watanabe, M. Wakimasu, M. Fujino, Biochem. Biophys. Res. Commun., 200, 1708 (1994).
98. S. Ikedda, Y. Awane, K. Kusumoto, M. Wakimasu, T. Watanabe, M. Fujino, J. Pharmacol. Exp. Ther., 270, 728 (1994).
99. K. Kusumoto, K. Kubo, H. Kandori, T. Kitayoshi, S. Sato, M. Wakimasu, T. Watanabe, M. Fujino, Life Sci., 55, 301 (1994).
100. N. Murugesan, Z. Gu, V. Lee, M.L. Webb, E.C.-K. Liu, M. Hermsmeier, J.T. Hunt, Bioorg. Med. Chem. Lett., 5, 253 (1995).

SECTION III. CANCER AND INFECTIOUS DISEASES

Editor: Jacob J. Plattner
Abbott Laboratories, Abbott Park, IL 60064

Chapter 11. Antibacterial Agents

John P. Dirlam, Burton H. Jaynes, and Martin R. Jefson
Pfizer Inc, Central Research Division, Groton, CT 06340

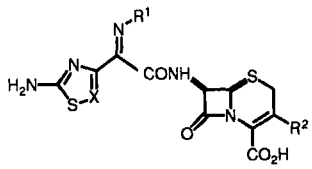
Introduction - The expanding prevalence of resistant bacteria and the possible consequences have been increasingly documented in the scientific literature and have attracted the attention of the general news media (e.g., 1-3). Response by the medical community, including government, academic, and industrial laboratories, has been to continue to search within known antibiotic classes for improved agents, look for novel classes lacking cross-resistance, and study resistance mechanisms in order to approach the problem in a rational manner. Reflecting the heightened interest, a new international conference was convened to address resistance emergence and its impact on discovery (4). This review summarizes important developments of the past year related to β-lactams, quinolones, tetracyclines, and macrolides, in addition to several less-developed areas that offer potential improvements in therapeutic options.

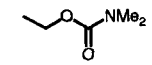
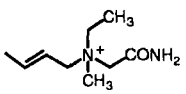
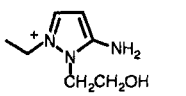
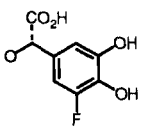
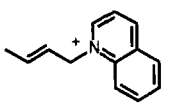
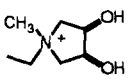
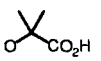
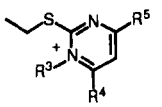
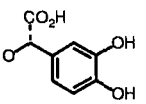
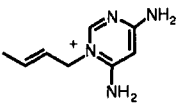
ß-Lactams - In the cephalosporin area, new information has appeared on compounds previously described in *Annu. Rep. Med. Chem.* E1101, the isopropoxycarbonyloxymethyl ester prodrug of E1100 (**1**), exhibits good oral absorption in laboratory animals (5) and humans (6), and has been the subject of an extensive chemistry and SAR study (7). Compound **1** displays potent activity against a variety of sensitive strains including respiratory infections caused by *K. pneumoniae* and *H. influenzae* and urinary tract infections caused by *E. coli*, but possesses poor activity against *P. aeruginosa*, methicillin-resistant *S. aureus* (MRSA), and *E. faecalis* (8). The therapeutic efficacy of parenteral agent E1077 (**2**) has been reported for a mixed respiratory tract infection caused by both *P. aeruginosa* and *S. aureus* in mice (9). Several studies have been conducted in laboratory models to ascertain its potential for uterine infections (10), and catheter related sepsis in burns (11). Published results on parenteral agent FK037 (**3**) include several comparative studies of *in vitro* activity with other cephalosporins (12-14), and a pharmacokinetic study in humans (15). Compound **3** displayed activity against *P. aeruginosa* that was similar to ceftazidime (MIC_{90} = 4 and 8 μg/ml, respectively).

A number of new cephalosporins reported in 1994 contain a catechol substituent in order to take advantage of the *tonB*-dependent iron transport system of bacteria. The SAR of a series of catechol-substituted vinylogous cephalosporins was described (16). RU-59863 (**4**), which is a very broad spectrum agent, showed impressive activity against MRSA, extended-spectrum β-lactamase strains of *Enterobacteriaceae*, and ceftazidime-resistant *P. aeruginosa* (17). After SC administration in rats, **4** had a similar plasma concentration, a longer half-life and a higher AUC compared to cefpirome (18). Placement of the catechol in the C-3 side chain, instead of in the oxime functionality, is also known to be compatible with potent activity, and now the effect of the introduction of hydroxy groups to quaternary aliphatic or alicyclic amines at C-3, as in **5**, has been investigated (19). In this study **5** was found to afford better activity than ceftazidime. The SAR of 3-[(amino-pyrimidiniumyl)thio]methyl cephalosporins, e.g., **6**, show that quarternized moieties have better antimicrobial activity than neutral pyrimidines (20). A related compound, LK-10517 (**7**), has a broad

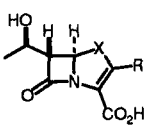
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

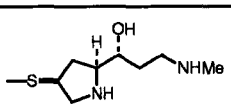
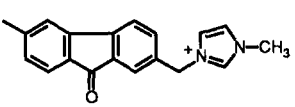
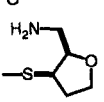
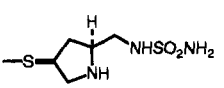
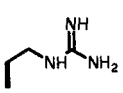
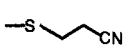
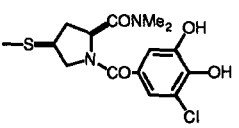
spectrum of activity including activity against systemic infections of *P. aeruginosa*, but it has poor activity against MRSA and *E. faecalis* (21).



	X	R^1	R^2
1	CH	OH	
2	N	OCH_2F	
3	CH	OCH_3	
4	CH		
5	CH	OCH_3	
6	CH		
7	CH		

The use of carbapenems in clinical practice was reviewed (22). Many carbapenems now contain the 1-β-methyl group that has been found to impart good stability to renal dehydropeptidase I (23). Of compounds previously described in *Annu. Rep. Med. Chem.*, BO-2727 (**8**) has been the subject of a number of recent publications, including a summary of its potent parenteral activity (ED_{50} = 19 mg/kg vs. high-level resistant MRSA and ED_{50} = 3.3 mg/kg vs. *P. aeruginosa* in mouse studies) (24), and a pharmacokinetic study in healthy male volunteers (25).

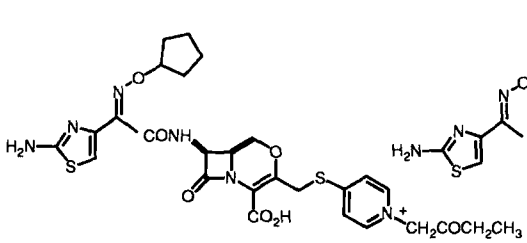


	X	R
8	CH-β-CH_3	
9	CH_2	
10	CH-β-CH_3	
11	CH-β-CH_3	
12		
13	CH-β-CH_3	

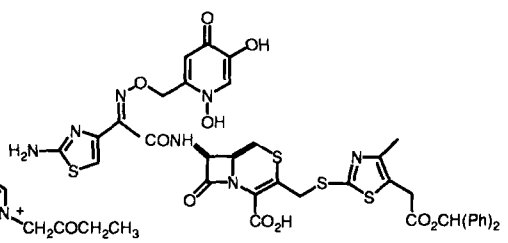
New members of the carbapenem class that are stable to hydrolysis by DHP-I include L-695256 (**9**), selected from a series of novel 2-fluorenonyl analogs with excellent activity against resistant Gram-positive organisms, including MRSA (26).

Another stable series of tetrahydrofuran-substituted penems, as in CL-191121 (**10**), showed excellent broad spectrum activity, although deficient against MRSA (27). S-4661 (**11**) and BMS-181139 (**12**), which are both stable to DHP-I, possess a broad spectrum of activity as well as being particularly effective against *P. aeruginosa* (28,29). The successful attachment of catechol units to carbapenems has been reported, e.g. **13**, and has provided a broad spectrum and enhanced potency, especially against *P. aeruginosa* (30).

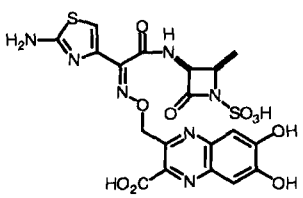
A number of reports appeared describing β-lactams with more unusual structures. A convenient synthesis of 2-oxaisocephems with (N-alkylpyridinium-4'-thio)methyl groups at the 3-position, e.g. **14**, has been published (31). Compound **14** displays potent activity against MRSA, and is eleven times as effective as vancomycin in a mouse high-resistant MRSA systemic infection model. A series of 2-isocephems, such as **15**, shows excellent anti-pseudomonal *in vitro* potency and *in vivo* activity in mice (32). A new catechol-containing monobactam, BM-180680 (**16**), with a directed Gram-negative spectrum, has been evaluated for *in vivo* efficacy in a variety of experimental bacterial infections as well as localized kidney and lung infections in mice (33). Compound **16**, which had favorable pharmacokinetic profiles in mice and rats with both a urinary and biliary route of excretion, could prove of value in the therapy of serious Gram-negative infections in man (i.e., *Enterobacteriaceae*, *Pseudomonas*, *Acinetobacter* and *P. mirabilis*). GV-118819 (**17**) demonstrated better efficacy orally in a mouse model of *S. pneumoniae* in comparison to positive controls such as ciprofloxacin and amoxicillin/clavulanate (34).



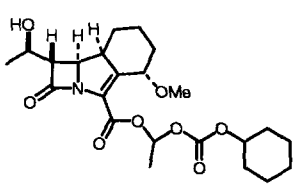
14



15

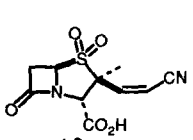


16

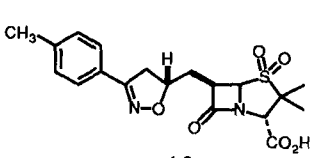


17

Recently, β-lactamase inhibitors have been reviewed as targets for drug design (35). In this area of research, Ro-48-1220 (**18**) is the most potent member within a new series of 2-β-alkenyl penam sulfones. Compound **18**, in synergy with apalcillin, compared favorably with currently marketed inhibitor combinations against a number of penicillinase- and cephalosporinase-producing bacteria (36). CL-186195 (**19**) represents a novel class of β-lactamase inhibitors with improved activity for Group 1 and 3 β-lactamases (37).



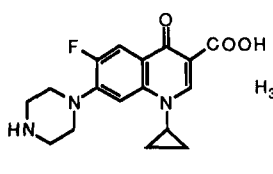
18



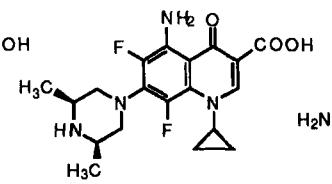
19

Quinolones - The recent literature on newer quinolones chronicles an intense search for compounds with improved potency relative to ciprofloxacin (**20**) and ofloxacin against the Gram-positive cocci, anaerobes, and quinolone-resistant MRSA, *Pseudomonas aeruginosa* and the *Enterobacteriaceae*. The antimicrobial and pharmacokinetic properties of most of the quinolones under development including sparfloxacin (**21**), clinafloxacin (**22**), PD-131682, PD-138312 (**23**), PD-140248, Q-35, AM-1155 (**24**), NM394, T-3761, rufloxacin, levofloxacin, CP-99219 (**25**), OPC-17116 (**26**) and DU-6859a (**27**) have been reviewed (38,39). New information pertaining to the mechanisms of quinolone activity and toxicity has also appeared. Topoisomerase IV may be a target for the quinolones in intact bacteria (40), and accumulation of quinolones is significantly increased in postantibiotic effect (PAE) phase cells relative to actively growing cells (41). The potency of quinolones against mammalian topoisomerase II is highly dependent on the structure of the C-7 substituent (42). The mechanism of quinolone-related arthropathy (43) and adverse effects on the central nervous system have also been examined (44).

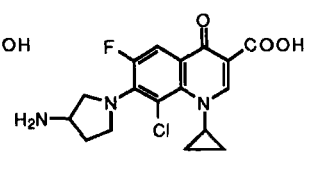
Substantial new information has been published for several of the agents cited above (38). The synthesis, antibacterial activity, pharmacokinetics, metabolism and safety of DU-6859a (**27**) has been reviewed (45). Numerous new reports on the antibacterial activity of **27** have also issued (46-53). In comparative studies, the potency of **27** against Gram-positive cocci, anaerobes and mycobacteria was superior to that of all other quinolones tested. However, studies comparing the activity of **27** and vancomycin against MRSA have yielded variable results (46). Against *E. coli, P. aeruginosa* and chlamydia, the activity of **27** was comparable to that of **20**. An SAR study found **27** to be the most potent compound among eight possible stereoisomers (54), and an extended PAE was demonstrated for **27** against *E. coli* and *S. aureus* (55).



20



21

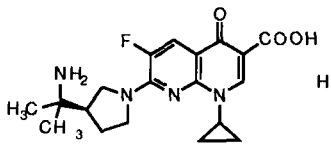


22

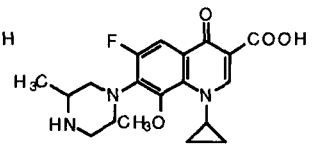
CP-99219 (**25**) has also been reported to have excellent activity against Gram-positive and Gram-negative bacteria (56-58) as well as anaerobes (59,60). Compound **25** is more potent than either **20** or **21** against quinolone- and methicillin-resistant staphylococci, pneumococci, enterococci and anaerobes, and comparable to **21** against *P. aeruginosa* and the *Enterobacteriaceae*. Although **25** is somewhat less potent *in vitro* than **27** against both Gram-positive and Gram-negative organisms (61), the pharmacokinetics of **25** are superior in humans following oral dosing (38). Compound **25** was highly potent against *Helicobacter pylori in vitro* and was efficacious as a single agent in an *H. felis* mouse model (62). Additionally, **25** was shown to be extensively distributed into the gastric mucosa of rats and mice (63). CP-116,517 (**28**), an effective L-ala-L-ala-prodrug of **25**, is being advanced as an intravenous companion to **25** (64).

OPC-17116 (**26**) showed a high distribution into the lung tissue of rodents along with potent activity against important respiratory tract pathogens (65). Compound **26** is equipotent to **20** against anaerobes (66) and slightly inferior to **21** against chlamydia (67). However, **26** is not one of the most potent new quinolones against anaerobes (38), and it appears that certain resistance determinants in *S. aureus* affect it to a much greater extent than other quinolones (68). PD-138312 (**23**) is

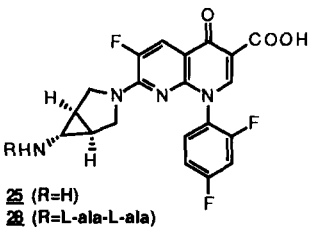
reported to have exceptional oral efficacy against Gram-positive pathogens and a favorable side-effect profile in comparison to a group of close-in analogs (69), but was inferior to PD-131682 against *Xanthomonas maltophilia* (70). AM-1155 (**24**) showed comparable potency to **21** against a broad range of pathogens (71). Bay y 3118 displayed potency superior to **20** against Gram-negative and Gram-positive bacteria (72) and anaerobes (73), but is reported to have a safety profile that is inconsistent with widespread use (38).



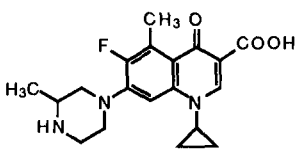
23



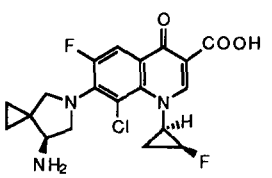
24



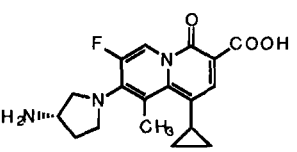
25 (R=H)
26 (R=L-ala-L-ala)



26



27



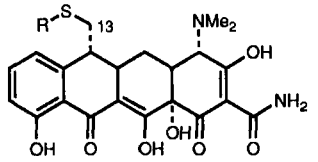
29

The pyridopyridones, a novel class of DNA gyrase inhibitors exemplified by A-86719.1 (**29**), were reported at the 34th ICAAC (74). Compound **29** displayed high potency *in vitro* (all MIC_{90}s ≤ 2 μg/ml) against a broad spectrum of Gram-negative and Gram-positive bacteria and anaerobes. In comparative studies **29** was equivalent to or two-fold more active than **22** against all isolates tested, and **29** was two-fold more active than **27** against most Gram-positive organisms. Compound **29** was found to be rapidly bactericidal with an exceptionally long PAE, and was more highly effective *in vivo* than **20** against lung, abscess or systemic infections caused by Gram-positive or Gram-negative bacteria, and vancomycin against enterococcal infections in mice.

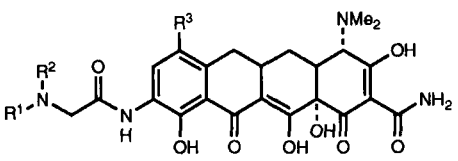
Tetracyclines - The increased need for new agents for combating antibiotic-resistant bacteria has rejuvenated interest in the tetracycline class. Two common mechanisms of resistance to tetracyclines exist: 1) removal of drug from the bacterial cell by membrane-bound efflux proteins and 2) ribosomal protection that prevents tetracyclines from interrupting polypeptide chain elongation. One of the proteins involved in the former mechanism, Tet A, is expressed when tetracycline binds to Tet repressor. A 2.5 Å resolution crystal structure of Tet repressor complexed with tetracycline-magnesium revealed detailed drug interactions and suggested its inability to bind to the DNA operator was due to a widening of the DNA binding domain (75). A study of structural requirements for inhibition of the efflux protein and ability to block active efflux of tetracyclines in whole cells found 13-(alkylthio)-tetracycline derivatives (**30**) to be potent inhibitors (76).

The glycylcyclines represent the newest members of the tetracycline class and have been found to have broad spectrum activity against tetracycline sensitive and resistant strains containing ribosomal protection (Tet M and O) and efflux (Tet A through E, L, and K) determinants. These 9-(substituted glycylamido)-tetracyclines have been reported in the sancycline, minocycline, and doxycycline series, including some examples of 8-chloro analogs (77-79). Antibacterial activity has been demonstrated with Gram-positive and Gram-negative pathogens including strains

resistant to β-lactams, minocycline, and vancomycin (80-85). Three analogs (DMG-Mino (**31**), TBG-Mino (**32**), and DMG-DMDOT (**33**)) have protected mice against a variety of characterized tetracycline-resistant challenge strains following IV or SC dosing, but are less effective PO (86). Compound **33** inhibits protein synthesis much like classical tetracyclines, but by virtue of the 9-dimethylglycyl amide is able to circumvent both efflux and ribosomal protection mechanisms (87,88). Transport studies suggest that while **33** still induces *tetA* gene expression, the 9-substituent prevents recognition of the compound by the Tet A efflux protein (89).

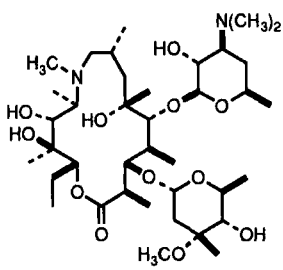


30

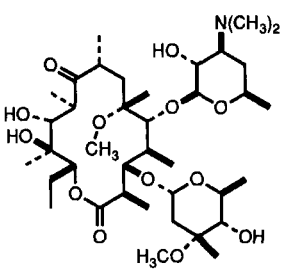


31: $R^1 = R^2 = Me$, $R^3 = NMe_2$
32: $R^1 = t\text{-}Bu$, $R^2 = H$, $R^3 = NMe_2$
33: $R^1 = R^2 = Me$, $R^3 = H$

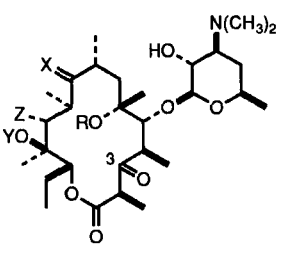
Macrolides - Several general reviews of macrolide chemistry and biology have appeared over the last year (90,91). Reports summarizing the activity and therapeutic utility of four recently introduced macrolides, i.e., azithromycin (**34**), clarithromycin (**35**), dirithromycin, and roxithromycin, were published (92-95). A significant number of studies have addressed *in vitro* and *in vivo* activities of these agents, especially azithromycin and clarithromycin, against opportunistic mycobacteria (96-100), *Toxoplasma gondii* (101,102), and *P. aeruginosa* (103). The well-precedented ability of macrolides to penetrate and accumulate in various host cells has led to: studies of the utility of macrolides versus infections caused by intracellular pathogens (e.g., chlamydia (104-107)) and enteric bacteria (108); and further studies on uptake and effect on macrophages, neutrophils, and T-lymphocytes (109-111). Multicenter clinical trials, with supporting *in vitro* studies, have demonstrated the utility of the new macrolides against bacterial pneumonia (112-114). Clarithromycin was found to bind tightly to isolated ribosomes from *H. pylori*, thus confirming the possible role of this macrolide in treating gastric disease in which *H. pylori* is implicated (115).



34



35

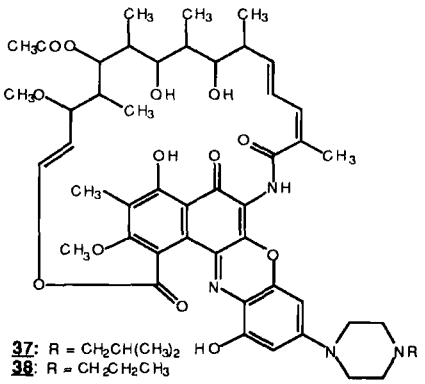


36

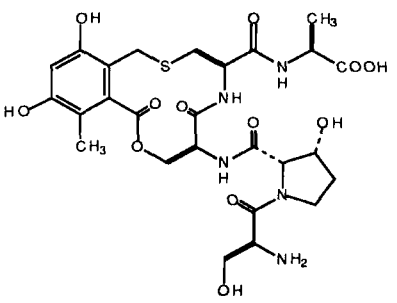
Interesting new macrolides based on both 14- and 16-membered templates were reported in 1994. A new class of macrolides, the ketolides (**36**), in which the cladinose sugar is replaced by a ketone, was synthesized and demonstrated potent *in vitro* activity against a range of multi-resistant pathogens (116). For example, ketolides were active against penicillin- and erythromycin-resistant pneumococci and *H. influenzae*. Oral mouse efficacy was observed against these organisms. This class of macrolides shows high acid stability owing to the lack of the cladinose moiety. Structure-activity relationships were reported for 9-oxime analogs in both the clarithromycin (117) and oleandomycin series (118). In the 16-membered macrolide

domain, polyhydrotylosin analogs (119), a C-2 methylated 3,4'-dideoxy-5-*O*-mycaminosyltylonolide (120), and putative izenamicin biosynthetic precursors (121) were reported. None offered biological advantages over the parent structures.

Miscellaneous - The glycopeptide antibiotics vancomycin and teicoplanin are used extensively to treat nosocomial infections caused by multi-resistant staphylococci and enterococci, thus the emergence of resistance to glycopeptides among these strains is of great concern to the medical community (122-124). Substantial effort has been undertaken during the past decade to identify glycopeptides with improved properties, and this work has been recently summarized (125). New work on glycopeptides has also been published. Balhimycin, a new glycopeptide, shows improved potency against *Clostridium* strains and better bactericidal activity and PAE compared to vancomycin (126). Semisynthetic modifications of eremomycin, vancomycin and teicoplanin targeting compounds with improved spectrum and potency against resistant isolates have also been reported (127-129). RP-59500, a synergistic mixture of two semisynthetic streptogramins, has also shown promise against vancomycin-resistant pneumococci and enterococci (130-132). Several new reports have issued on the rifamycin derivatives KRM-1648 (**37**) and KRM-1657 (**38**). Neither **37** nor **38** showed any advantages over rifamycin against bacteria (133), but **37** looks quite promising as an antimycobacterial, exhibiting good activity against *M. avium* and *M. tuberculosis* infections in mice (134-136).



37: R = $CH_2CH(CH_3)_2$
38: R = $CH_2CH_2CH_3$



39

From screening of natural products for DNA gyrase inhibitors, a novel gyrase inhibitor, cyclothialidine (Ro 09-1437, **39**), was isolated from *Streptomyces filipinensis* NR0484 (137,138). Mechanistic studies suggest that **39** is a B-subunit ATPase inhibitor with a mechanism more similar to the coumarin-type antibiotics than to A-subunit inhibitor quinolones (139). However, cyclothialidine was active against coumarin-resistant DNA gyrase isolated from an *E. coli gyrB* mutant, indicating some differences in the specific site of action. Compound **39** is a very potent inhibitor of bacterial DNA gyrase, yet shows only weak activity against mammalian DNA topoisomerase II (140). Cyclothialidine has been found to be minimally active against a variety of aerobic and anaerobic bacteria, and poor cytoplasmic membrane penetration has been implicated. A chemical modification program defined minimal structural requirements, provided analogs with improved *in vitro* antibacterial activity, especially against Gram-positive bacteria, and led to a total synthesis of the natural product (141).

References

1. E. Rubinstein, Ed., Science, 264, 360 (1994).
2. S. Begley, Newsweek, March 28, p. 46 (1994).
3. W. Brumfitt and J.M.T. Hamilton-Miller, Drugs Exptl. Clin. Res., 20, 215 (1994).
4. J.F. Barrett, Exp. Opin. Invest. Drugs, 3, 1227 (1994).

5. 34th ICAAC (1994), Abs. Nos. F85 and F87.
6. M. Nakashima, T. Uematsu, K. Umemura, K. Kosuge, S. Yasuda, M. Kawahara, Y. Tomono, T. Ohno, K. Okano and C. Yamato, 34th ICAAC, F89 (1994).
7. S. Negi, M. Yamanaka, I. Sugiyama, Y. Komatsu, M. Sasho, A. Tsuruoka, A. Kamada, I. Tsukada, R. Hiruma, K. Katsu and Y. Machida, J. Antibiot., 47, 1507 (1994).
8. 34th ICAAC (1994), Abs. Nos. F73, F77, F79 and F81.
9. 34th ICAAC (1994), Abs. Nos. F111 and F113.
10. 34th ICAAC (1994), Abs. Nos. F117, F119 and F121.
11. K. Takuma, N. Aikawa, Y. Shinozawa, S. Hori, J. Sasaki, S. Hachiya and M. Satoh, 34th ICAAC, F115 (1994).
12. S. K. Spangler, M. R. Jacobs and P. C. Appelbaum, Antimicrob. Agents Chemother., 38, 898 (1994).
13. R. Wise, J. M. Andrews and D. Thornber, J. Antimicrob. Chemother., 34, 629 (1994).
14. R. N. Jones, M. S. Barrett and M. E. Erwin, J. Antimicrob. Chemother., 34, 137 (1994).
15. R. Wise, J. M Andrews, P. O'Neill, A. Jolley, C. Fowler and M. C. Rogge, Antimicrob. Agents Chemother., 38, 2369 (1994).
16. 34th ICAAC (1994), Abs. Nos. F95 and F97.
17. J. Aszodi, D. Humbert, A. Bonnefoy, P. Collette, P. Mauvais and J. F. Chantot, 34th ICAAC, F99 (1994).
18. J. Aszodi, A. Bonnefoy and J. F. Chantot, 34th ICAAC, F101 (1994).
19. Y. S. Lee, J. Y. Lee, S. H. Jung, E.-R. Woo, D. H. Suk, S. H. Seo and H. Park, J. Antibiot., 47, 609 (1994).
20. Y.-Z. Kim, J.-C. Lim, J.-H. Yeo, C.-S. Bang, W.-S. Kim, S.-S. Kim, Y.-M. Woo, D.-H. Yang, H. Oh and K. Nahm, J. Med. Chem., 37, 3828 (1994).
21. J. H. Kwak, J. I. Oh, H. K. Song, S. I. Kim, M. Y. Kim, J. H. Song, M. K. Seo, Y. N. Jeong, M. H. Cho, Y. H. Lee, Y. Z. Kim and I. C. Kim, 34th ICAAC, F93 (1994).
22. G. L. Drusano, Ed., Curr. Opin. Infect. Dis., 7, Suppl. 1, S-1 (1994).
23. M. Hikida, K. Kawashima, M. Yoshida and S. Mitsuhashi, J. Antimicrob. Chemother., 30, 129 (1992).
24. 34th ICAAC (1994), Abs. Nos. F1, F3, F5, F7, F9, F11, F13, F15, F17, F19 and F21.
25. M. Nakashima, T. Uematsu, K. Kosuge, S. Nakagawa, S. Hata and M. Sanada, J. Antimicrob. Chemother., 34, 987 (1994).
26. 34th ICAAC (1994), Abs. Nos. B85, F50, F52, F54, F56, F58, F60, F62, F64 and F66.
27. 34th ICAAC (1994), Abs. Nos. F72, F74 and F78.
28. 34th ICAAC (1994), Abs. Nos. F33, F35, F37 and F39.
29. 34th ICAAC (1994), Abs. Nos. F42, F44 and F48.
30. M. Sunagawa, A. Sasaki, H. Yamaga, H. Shinagawa, M. Fukasawa and Y. Sumita, J. Antibiot., 47, 1354 (1994).
31. H. Ishikawa, H. Tsubouchi and K. Yasumura, BioMed. Chem. Lett., 4, 1147 (1994).
32. K. Tsuji and H. Ishikawa, BioMed. Chem. Lett., 4, 1601 (1994).
33. 34th ICAAC (1994), Abs. Nos. F143 and F145.
34. 34th ICAAC (1994), Abs. Nos. F80 and F82.
35. S. Coulton and I. Francois, Prog. Med. Chem., 31, 297 (1994).
36. 34th ICAAC (1994), Abs. Nos. F147, F149, F151, F153 and F155.
37. C. B. Ziegler, Jr., Y. Yang, P. Fabio, D. A. Steinberg, W. Weiss, M. J. Wildey and K. Bush, 34th ICAAC, C54 (1994).
38. T. D. Gootz and P. R. McGuirk, Exp. Opin. Invest. Drugs, 3, 93 (1994).
39. L. J. V. Piddock, Antimicrob. Agents Chemother., 38, 163 (1994).
40. K. Hoshino, A. Kitamura, I. Morrissey, K. Sato, J.-I. Kato and H. Ikeda, Antimicrob. Agents Chemother., 38, 2623 (1994).
41. R. J. Davidson, G. R. Drobot, J. A. Karlowsky, G. G. Zhanel, R. Philips and D. J. Hoban, J. Antimicrob. Chemother., 34, 363 (1994).
42. T. D. Gootz, P. R. McGuirk, M. S. Moynihan and S. L. Haskell, Antimicrob. Agents Chemother., 38, 130 (1994).
43. G. Hayem, P. X. Petit, M. Levacher, C. Gaudin, M.-F. Kahn and J.-J. Pocidalo, Antimicrob. Agents Chemother., 38, 243 (1994).
44. K. Akahane, Y. Kimura, Y. Tsutomi and I. Hayakawa, Antimicrob. Agents Chemother., 38, 2323 (1994).
45. J. Prous, A. Graul and J. Castaner, Drugs of the Future, 19, 827 (1994).
46. 34th ICAAC (1994), Abs. Nos. F57, F61, F63, F65, F67, F69 and F71.
47. S. A. Marshall and R. N. Jones, Antimicrob. Agents Chemother., 37, 2747 (1993).
48. A. Jolley, J. M. Andrews, N. Brenwald and R. Wise, J. Antimicrob. Chemother., 32, 757 (1993).
49. S. A. Marshall, R. N. Jones, P. R. Murray, J. A. Washington, S. D. Allen, E. H. Gerlach and M. E. Erwin, J. Antimicrob. Chemother., 32, 877 (1993).
50. J. F. Tomayko, V. Korten and B. E. Murray, Diagn. Microbiol. Infect. Dis., 20, 45 (1994).
51. V. Korten, J. F. Tomayko and B. E. Murray, Antimicrob. Agents Chemother., 38, 611 (1994).
52. H. Saito, H. Tomioka, K. Sato and S. Dekio, Antimicrob. Agents Chemother., 38, 2877 (1994).
53. H. M. Wexler, E. Molitoris, D. Reeves and S. M. Finegold, Antimicrob. Agents Chemother., 38, 2504 (1994).

54. Y. Kimura, S. Atarashi, K. Kawakami, K. Sato and I. Hayakawa, J. Med. Chem., 37, 3344 (1994).
55. A. K. Houston and R. N. Jones, Diagn. Microbiol. Infect. Dis., 18, 57 (1994).
56. H. C. Neu and N.-X. Chin, Antimicrob. Agents Chemother., 38, 2615 (1994).
57. T. D. Gootz, K. E. Brighty, M. R. Anderson, B. J. Schmieder, S. L. Haskell, J. A. Sutcliffe, M. J. Castaldi and P. R. McGuirk, Diagn. Microbiol. Infect. Dis., 19, 235 (1994).
58. 34th ICAAC (1994), Abs. Nos. F2, F4, F6, F8, F10, F12, F14, F16 and F18.
59. S. K. Spangler, M. R. Jacobs and P. C. Appelbaum, Antimicrob. Agents Chemother., 38, 2471 (1994).
60. S. K. Spangler, M. R. Jacobs and P. C. Appelbaum, 34th ICAAC, F20 (1994).
61. R. Wise, J. M. Andrews and N. Brenwald, 34th ICAAC, F22 (1994).
62. A. E. Girard, C. R. Cimochowski, D. Girard, T. D. Gootz and K. E. Brighty, 34th ICAAC, F24 (1994).
63. R. J. Polzer, M. J. Potchoiba, D. N. Renouf, M. West and T. E. Liston, 34th ICAAC, F30 (1994).
64. K. E. Brighty, T. D. Gootz, A. Girard, R. Shanker, M. J. Castaldi, D. Girard, S. A. Miller and J. Faiella, 34th ICAAC, F28 (1994).
65. H. Wakebe, T. Imada, H. Yoneda, F. Mukai, K. Ohguro, K. Ohmori, H. Tamaoka and Y. Yabuuchi, Antimicrob. Agents Chemother., 38, 2340 (1994).
66. H. M. Wexler, E. Molitoris and S. M. Finegold, Diagn. Microbiol. Infect. Dis., 19, 129 (1994).
67. P. M. Roblin, G. Montalban and M. R. Hammarschlag, Antimicrob. Agents Chemother., 38, 1402 (1994).
68. K. S. Thomson and C. C. Sanders, Antimicrob. Agents Chemother., 38, 2095 (1994).
69. S. E. Hagen, J. M. Domagala, S. J. Gracheck, J. A. Sesnie, M. A. Stier and M. J. Suto, J. Med. Chem., 37, 733 (1994).
70. G. A. Pankuch, M. R. Jacobs and P. C. Appelbaum, Antimicrob. Agents Chemother., 38, 369 (1994).
71. E. Wakabayashi and S. Mitsuhashi, Antimicrob. Agents Chemother., 38, 594 (1994).
72. L. J. V. Piddock, A. J. Marshall and Y.-F. Jin, Antimicrob. Agents Chemother., 38, 422 (1994).
73. K. E. Aldridge, Antimicrob. Agents Chemother., 38, 1671 (1994).
74. 34th ICAAC (1994), Abs. Nos. F41, F43, F45, F47, F49, F51, F53 and F55.
75. W. Hinrichs, C. Kisker, M. Düvel, A. Müller, K. Tovar, W. Hillen and W. Saenger, Science, 264, 418 (1994).
76. M.L. Nelson, B.H. Park and S.B. Levy, J. Med. Chem. 37, 1355 (1994).
77. P.-E. Sum, V.J. Lee, R.T. Testa, J.J. Hlavka, G.A. Ellestad, J.D. Bloom, Y. Gluzman and F.P. Tally, J. Med. Chem. 37, 184 (1994).
78. T.C. Barden, B.L. Buckwalter, R.T. Testa, P.J. Petersen and V.J. Lee, J. Med. Chem. 37, 3205 (1994).
79. P.-E. Sum, V.J. Lee, P.J. Petersen, N.V. Jacobus, R.T. Testa and F.P. Tally, 34th ICAAC, F84 (1994).
80. G.M. Eliopoulos, C.B. Wennersten, G. Cole and R.C. Moellering, Antimicrob. Agents Chemother., 38, 534 (1994).
81. R. Wise and J.M. Andrews, Antimicrob. Agents Chemother., 38, 1096 (1994).
82. F.W. Goldstein, M.D. Kitzis and J.F. Acar, Antimicrob. Agents Chemother., 38, 2218 (1994).
83. H.M. Wexler, E. Molitoris and S.M. Finegold, Antimicrob. Agents Chemother., 38, 2513 (1994).
84. G.E. Kenny and F.D. Cartwright, Antimicrob. Agents Chemother., 38, 2628 (1994).
85. 34th ICAAC (1994), Abs. Nos. F84, F90, F92, F94, F96, F98, F100 and F106.
86. 34th ICAAC (1994), Abs. Nos. F102, F104, F108 and F118.
87. B.A. Rasmussen, Y. Gluzman and F.P. Tally, Antimicrob. Agents Chemother., 38, 1658 (1994).
88. 34th ICAAC (1994), Abs. Nos. F88 and F110.
89. Y. Someya, A. Yamaguchi and T. Sawai, Antimicrob. Agents Chemother., 39, 247 (1995).
90. H. Kirst, Prog. Med. Chem., 31, 265 (1994).
91. P.A. Lartey, H.N. Nellans and S.K. Tanaka, Advances in Pharmacology, 28, 307 (1994).
92. J.D. Williams, Curr. Opin. Infect. Dis., 7, 653 (1994).
93. J. Winter, Br. J. Hosp. Med., 51, 295 (1994).
94. R.N. Brogden and D.H. Peters, Drugs, 48, 599 (1994).
95. A. Markham and D. Faulds, Drugs, 48, 297 (1994).
96. S.P. Klemens and M.H. Cynamon, Antimicrob. Agents Chemother., 38, 1721 (1994).
97. C.O. Onyeji, C.H. Nightingale, D.P. Nicolau and R. Quintiliani, Antimicrob. Agents Chemother., 38, 523 (1994).
98. L.E. Bermudez, C.B. Inderlied, P. Kolonoski, M. Petrofsky and L.S. Young, Antimicrob. Agents Chemother., 38, 2717 (1994).
99. N. Mor, J. Vanderkolk, N. Mezo and L Heifets, Antimicrob. Agents Chemother., 38, 2738 (1994).
100. 34th ICAAC (1994), Abs. Nos. E134, E136, E140, I189, I191, I193, I195, I197 and I199.
101. J. Blais, D. Beauchamp and S. Chamberland, J. Antimicrob. Chemother., 34, 371 (1994).
102. J.C. Schwab, Y. Cao, M.R. Slowik and K.A. Joiner, Antimicrob. Agents Chemother., 38, 1620 (1994).
103. R. Mizukane, Y. Hirakata, M. Kaku, Y. Ishii, N. Furuya, K. Ishida, H. Koga, S. Kohno and K. Yamaguchi, Antimicrob. Agents Chemother., 38, 528 (1994).

104. Y. Niki, M. Kimura, N. Miyashita and R. Soejima, Antimicrob. Agents Chemother., 38, 2296 (1994).
105. J.E. Raulston, J. Antimicrob. Chemother., 34, 765 (1994).
106. P.M. Roblin, G. Montalban and M.R. Hammerschlag, Antimicrob. Agents Chemother., 38, 1588 (1994).
107. J. Segreti and K.S. Kapell, Antimicrob. Agents Chemother., 38, 2213 (1994).
108. R.M. Rakita, K. Jacques-Palaz and B.E. Murray, Antimicrob. Agents Chemother., 38, 1915 (1994).
109. K. Morikawa, F. Oseko, S. Morikawa and K. Iwamoto, Antimicrob. Agents Chemother., 38, 2643 (1994).
110. H. Abdelghaffar, E.M. Mtairag and M.-T. Labro, Antimicrob. Agents Chemother., 38, 1548 (1994).
111. R.P. Baughman, K.A. DeSante, T.L. Lanier, P.M. Conforti and G.D. Sides, J. Antimicrob. Chemother., 33, 1045 (1994).
112. K. Ishida, M. Kaku, K. Irifune, R. Mizukane, H. Takemura, R. Yoshida, H. Tanaka, T. Usui, N. Suyama, K. Tomono, H. Koga, S. Kohno, K. Izumikawa and K. Hara, Antimicrob. Agents Chemother., 38, 790 (1994).
113. K. Liippo, E. Tala, H. Puolijoki, O.-J. Brückner, J. Rodrig and J.P.H. Smits, J. Infect., 28, 131 (1994).
114. S.W. Lemmen, K. Anding, I. Engels and F.D. Daschner, J. Antimicrob. Chemother., 33, 673 (1994).
115. R.C. Goldman, D. Zakula, R. Flamm, J. Beyer and J. Capobianco, Antimicrob. Agents Chemother., 38, 1496 (1994).
116. 34th ICAAC (1994), Abs. Nos. F164, F166 and F168.
117. Y. Kawashima, Y. Yamada, T. Asaka, Y. Misawa, M. Kashimura, S. Morimoto, T. Ono, T. Nagate, K. Hatayama, S. Hirono and I. Moriguchi, Chem. Pharm. Bull., 42, 1088 (1994).
118. G. Lazarevski, G. Kobrehel, S. Dokic, L. Kolacny-Babic, B. Kojic-Prodic, D. Jankovic and V. Puntarec, J. Antibiot., 47, 349 (1994).
119. A. Narandja, B. Suskovic, Z. Kelneric and S. Djokic, J. Antibiot., 47, 581 (1994).
120. S. Kageyama and T. Tsuchiya, J. Antibiot., 47, 955 (1994).
121. K. Yasumuro, M. Shibazaki, T. Sasaki, H. Imai, H. Yamaguchi, K. Suzuki, M. Morioka and Y. Takebayasi, J. Antibiot., 47, 250 (1994).
122. N. Woodford and A. P. Johnson, J. Med. Microbiol., 40, 375 (1994).
123. N. C. Clark, R. C. Cooksey, B. C. Hill, J. M. Swenson and F. C. Tenover, Antimicrob. Agents Chemother., 37, 2311 (1993).
124. M. J. Haverkorn, J. Infection, 27, 335 (1994).
125. D. Felmingham, J. Antimicrob. Chemother., 32, 663 (1993).
126. S. R. Nadkarni, M. V. Patel, S. Chatterjee, E. K. S. Vijayakumar, K. R. Desikan, J. Blumbach, B. N. Ganguli and M. Limbert, J. Antibiot, 47, 334 (1994).
127. A. Y. Pavlov, E. N. Olsufyeva, T. F. Berdnikova, I. V. Malkova and M. N. Preobrazhenskaya and G. D. Risbridger, J. Antibiot, 47, 225 (1994).
128. A. Y. Pavlov, T. F. Berdnikova, E. N. Olsufyeva, E. I. Lazhko, I. V. Malkova, M. N. Preobrazhenskaya, R. T. Testa and P. J. Petersen, J. Antibiot, 46, 1731 (1993).
129. A. Malabarba and R. Ciabatti, J. Med. Chem., 37, 2988 (1994).
130. G. A. Pankuch, M. R. Jacobs and P. C. Appelbaum, Antimicrob. Agents Chemother., 38, 2065 (1994).
131. C.C. Johnson, L. Slavoski, M. Schwartz, P. May, A. Shur, P. G. Pitsakis and M. E. Levison, 34th ICAAC, E9 (1994).
132. 34th ICAAC (1994), Abs. Nos. J155 and M66.
133. K. Fujii, A. Tsuji, S. Miyazaki, K. Yamaguchi and S. Goto, Antimicrob. Agents Chemother., 38, 1118 (1994).
134. L. E. Bermudez, P. Kolonoski, L. S. Young and C. B. Inderlied, Antimicrob. Agents Chemother., 38, 1844 (1994).
135. S. P. Klemens, M. A. Grossi and M. H. Cynamon, Antimicrob. Agents Chemother., 38, 2245 (1994).
136. C. B. Inderlied, L. Barbara-Burnham, M. Wu, L. S. Young and L. E. M. Bermudez, Antimicrob. Agents Chemother., 38, 1838 (1994).
137. J. Watanabe, N. Nakada, S. Sawairi, H. Shimada, S. Ohshima, T. Kamiyama and M. Arisawa, J. Antibiot., 47, 32 (1994).
138. T. Kamiyama, N. Shimma, T. Ohtsuka, N. Nakayama, Y. Itezono, N. Nakada, J. Watanabe and K. Yokose, J. Antibiot., 47, 37 (1994).
139. N. Nakada, H. Gmünder, T. Hirata and M. Arisawa, Antimicrob. Agents Chemother., 38, 1966 (1994).
140. N. Nakada, H. Shimada, T. Hirata, Y. Aoki, T. Kamiyama, J. Watanabe and M. Arisawa, Antimicrob. Agents Chemother., 37, 2656 (1993).
141. E. Goetschi, P. Angehrn, H. Gmuender, P. Hebeisen, H. Link, R. Masciadri and J. Nielsen, Pharmacol. Ther., 60, 367 (1993).

Chapter 12. Bacterial Virulence as a Potential Target for Therapeutic Intervention

John F. Barrett[1] and Richard E. Isaacson[2]

The R. W. Johnson Pharmaceutical Research Institute[1], Raritan, NJ 08869
and the University of Illinois[2], Urbana, IL 61801

Introduction - Resistance to antibacterials/antibiotics has been a problem in the clinic for the last 10 years. Few would have predicted the high frequency of clinical failures due to resistance. With more than 50 antibacterials/antibiotics on the market currently, why are there serious problems due to methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci*, multiply-resistant *Mycobacterium tuberculosis*, and penicillin-resistant *Streptococcus pneumonia* (1)? There are no absolute answers to this question, although many of the contributing factors include overuse of antibacterials/antibiotics, inappropriate use of antibacterials/antibiotics, emergence of resistance "cassettes" within extrachromosomal elements capable of movement in the bacterial gene pool, and nature's inevitable ability to select for mutant survivors of any antibacterial/antibiotic thrown at them. So while the phenomenon must be dealt with if antibacterial therapy is to remain a viable part of the physician's arsenal, the exact causes for resistance emergence may be speculative. Regardless of the causes, antibacterial/antibiotic resistance is inevitable.

Perhaps a more important question is how to deal with the problems of resistance in antibacterials/antibiotics? Approaches could include incrementally improved drugs developed by increasing specific activities in known compounds. For example, increasing resistance to β-lactamase, the inclusion of a β-lactamase inhibitor to protect a β-lactam, a fluoroquinolone with better activity against gram-positive bacteria, or a macrolide with better tissue accumulating activity are approaches that will keep us just one step ahead of resistance emergence and all are threatened in the short term by the same resistance emergence factors that plagued their predecessors.

What alternatives to existing therapy or medical practices do we have in our repertoire? Examples include identification of better targets, novel targets, more cidal drugs, better combinations of therapy, or better dosing regimens. Indeed all of these represent reasonable approaches that need more thorough evaluation, but all are potentially subject to the same resistance problems.

With the antibacterial/antibiotic crisis now upon us (Table 1), there appears to be a movement to discover fundamentally different approaches to therapy that have their roots in microbiology. The fundamental precepts of the geneticists, the microbiologists, and the microbial physiologists relative to the importance of virulence and the ability of the bacteria to survive in the host have resurfaced in the literature during the last five years, especially with the availability of the new tools of molecular biology (2,3). Key to this understanding is the recent acknowledgment that bacteria express different genes when grown *in vitro* in the test tube when compared to life in an infected mammalian host (4,5).

Virulence Through the Eyes of Bacteria - The production of disease requires the expression of a series of important genes that allows the pathogen to adapt to hostile environment in the host (4). The expression of these genes contributes to the virulence of pathogens and such genes encode products frequently termed virulence factors. In the traditional sense, virulence factors are thought to include toxins attachment factors, and hydrolytic enzymes that contribute directly to

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Table 1. Crisis Organisms and Resistance Problems

Organism	Disease	Resistance Problems
S. aureus (MRSA)	systemic infections	multiply-resistant
Enterococci	systemic infections	vancomycin, teicoplanin
S. pneumococci	respiratory infections	penicillin, cephalosporins
M. tuberculosis	respiratory infections	multiply-resistant
P. aeruginosa	respiratory, skin infections	multiply-resistant
Enterobacteriaceae	abdominal infections	multiply-resistant
Bacteroides	abdominal infections	cefotetan, cefoxitin, metronidazole
Acinetobacter	abdominal infections	β-lactams
Streptococci	respiratory, skin infections	β-lactams

disease. For example, cell surface structures have been characterized in bacteria that permit both attachment and entry into epithelial cells (6), and pathogenic bacteria may use any of a number of proteinaceous (protein-cleaving) virulence factors to infect the host through the respiratory, gastrointestinal or genitourinary tract (7). Likewise, group A streptococci use lipoteichoic acid (LTA) and/or M proteins to facilitate attachment and infection (8). However, from the standpoint of the bacterium, virulence factors contribute to the ability of the organism to survive and grow at the site of infection, and thus in an ecological sense, virulence contributes to how an organism propagates in a mammalian host.

Furthermore, most pathogens have evolved highly sophisticated mechanisms to regulate the expression of virulence genes. Pathogens grown in non-mammalian habitats including culturing in the laboratory, tend not to express genes associated with disease since the products of these genes are not required for the survival of the organism in these environments. Mechanisms that "sense" the components in the environment that are responsible for the expression of genes required to cope with the current conditions allow bacteria to produce only those products required for survival and/or growth.

Given this perspective, the role of traditional virulence factors should be considered within the context of their role(s) in survival and growth of the organism. For example, lytic enzymes (such as hemolysins) probably are important in the acquisition of nutrients including iron. Using this perspective, the definition of virulence factors can be expanded to include the products of genes that may not necessarily contribute to the production of clinical signs and/or lesions, but contribute to the array of products required to be able to cope with the metabolic substrates (or lack of substrates) provided in the mammalian habitat. For example, these products could include enzymes required to metabolize complex proteins and glycoproteins found in connective tissues or blood. Following on from this viewpoint, it is possible to divide virulence factors into several distinct classes: traditional virulence factors (e.g. toxins), metabolically required factors, factors that contribute to the evasion of the host immune response, and genetic mechanisms that sense the environment.

Conventional thinking regarding the selection of targets to develop antimicrobial agents generally has been to identify agents that kill microorganisms *in vitro* with the expectation that these agents will be effective *in vivo*. Less conventional approaches have been to target known traditional virulence factors. For example, adhesins (bacterial recognition proteins) are important for the initial colonization of mucosal habitats which is a prerequisite for disease (9). The interference with the colonization process leads to reduction of disease. This observation has led to investigative studies to alter colonization by affecting the adhesin (10). Commercially available animal health products exploit this approach

through a vaccination tactic that uses the adhesin. Antibodies produced by vaccination neutralize the adhesin and thus prevent colonization.

Growth Inhibition or Killing of Bacteria - The studies on adhesins demonstrate the concept that the efficacy of anti-infective agents need not be tied to the killing of the microbe (10). The distinction between killing of bacteria and slowing or stopping growth is no longer an issue of semantics. The importance of "virulence antigens" in the development of vaccines has been recognized and exploited for many years (11). This concept has provided proof of the importance of these virulence antigens as vaccine antigens to successfully prevent disease. Most vaccines (whether deliberate of not) either inhibit colonization of the host by the pathogen or prevents pathologic effects generally by inhibiting a toxin. If the development of a vaccine to a non-obligate antigen such as pili of *Escherichia coli* prevents colonization, then a pharmacologic approach might be to use compounds that stop the production of these pili. The inhibition of virulence factors(s) would not kill the *Escherichia coli per se*, but rather would stop the initial colonization and subsequent infection. Table 2 lists some examples of the successful use of cell surface or virulence antigens as targets for intervention through vaccine development (recently reviewed in 12), and virulence antigens under study as potential vaccines (13). Such a strategy has also been applied to the antiviral arena, but has not been successfully applied to the area of antifungals.

Table 2. Successful Vaccine Therapy Based on Virulence Factors

Organism	Antigen/Virulence factor	Disease
Cornybacterium diphtheriae	diphtheria toxin	Diphtheria
Clostridium tetani	tetanus toxin	Tetanus
Vibrio cholerae	cholera toxin	Cholera
Salmonella typhi	surface antigens	Typhoid fever
Bordetella pertussis	pertussis toxin, filamentous hemagglutin	Pertussis
Streptococcus pneumoniae	capsule	Pneumoniae
Neisseria meningitidis	capsule	Meningitis
Haemophilus influenza	capsule	Meningitis
HIV	gp120	AIDS
Poliovirus	colonization	Polio
Influenza virus	colonization	Influenza

Empirical Therapy at Sub-MIC Levels - The use of sub-inhibitory doses of antimicrobials *in vitro* has led to some surprising results on bacterial virulence and bacterial growth (14). Six antibiotics (ciprofloxacin, enoxacin, chloramphenicol, gentamicin, tetracycline, and methicillin) were examined for their effects on the virulence (coagulase, protein A, alpha and delta hemolysin production and growth) of *S. aureus*. At sub-MIC levels of these agents different antibacterials inhibited different virulence factors, indicating a sub-MIC effect on virulence, distinct from their growth-inhibitory activities (15). Likewise, roxithromycin has shown a sub-MIC effect on gram-positive cocci (*S. aureus*, *Streptococcus pyogenes*, *S. pneumoniae*) by increasing the level of staphylococcal coagulase and DNase, leaving the α-hemolysin, streptolysins O and S unaltered, but lowering the pneumococcal capsule (16). Erythromycin has been shown to produce various effects on *P. aeruginosa*, distinct from the antimicrobial effect, suggesting that it may have a role in suppressing virulence or immunomodulation (17). Low concentrations of quinolones have been reported as reducing or eliminating certain virulence factors in bacteria (*Staphylococcus*, *E. coli*, *P. aeruginosa*, *S. typhimurium*)(18,19). Such effects included inhibition of bacterial nuclease, pili, fimbriae, adherence, phagocytosis, exocellular enzymes and plasmid maintenance (18). An inhibitor (8-amino-2,6-anhydro-3,8-dideoxy-D-glycero-D-talo-octonic acid) of lipopolysaccharide biosynthesis in *E. coli* has been reported to increase the susceptibility of *E. coli* to

hydrophobic antibiotics, complement-mediated antibacterial activity in the serum, detergents, phagocytosis, and clearance of the bacteria the mouse bloodstream at sub-MIC levels (20). Sub-inhibitory doses of rifampicin, chloramphenicol, tetracycline and nalidixic acid on the interactions of *E. coli* and *Shigella flexneri* 2a with HeLa cells demonstrated a decrease of virulence (adhesion, penetration, intracellular multiplication) with all antimicrobial agents except chloramphenicol (21). Similarly, extracts from the Kenyan chewing stick has been shown to effect virulence by inhibiting known virulence factors of periodontal gingivitis-causing bacteria (*Porphyromonas gingivalis, Bacteroides intermedius, Treponema denticola*) (22-24). The observations that certain non-antibiotic drugs have antibacterial activities *in vivo* have been recently reviewed (25).

The effects of sub-inhibitory levels of antibiotics described above may contribute to the effectiveness of traditional antibiotics and may contribute to the post-antibiotic effects observed for many classes of antibiotics. For the examples cited above, the antimicrobial effects alters the environmental location where the organism will reside. Clearly, many pathogenic bacteria, particularly those associated with opportunistic infections, also are components of the normal microflora of mammals (5). Whether these organisms cause disease is directly related to their habitat and to the immune status of the host. Consequently, approaches that alter the environmental distribution of disease-causing organisms should have anti-infective properties.

A bonus of this approach is that since the therapeutic outcome does not have to be total eradication of the organisms, but altered distribution, genetic pressure to resist the process will be reduced. This should lead to a reduced risk of resistance emergence.

Discovery of Metabolically Required Factors - The survival of bacteria in a given habitat in the infected host requires the expression of various gene products. Some of these gene products are expressed in all habitats and it is likely that inhibiting them will result in cell death. However, an emerging belief is that there are unique genes that may be expressed only *in vivo* and then only in specific tissues (26). Inhibition of these products should only affect the organism in this specific site (the site of infection) and the identification of these products as new targets to develop antibiotics is attractive. These targets are attractive because they also should represent targets not previously exploited.

Two breakthrough technologies have been developed that have permitted investigators to discover these new genes. The first method employs a powerful gene cloning/selection procedure based on finding gene sequences that stimulate the expression of a selective reporter gene (either a gene required for a specific metabolic purpose or agent encoding resistance to an antibiotic)(26). By isolating and sequencing these genetic elements their identity and their relevance as new targets can be determined. The second procedure employs subtractive hybridization using RNA isolated from infected tissues. RNA from laboratory grown bacteria and from non-infected tissues are used to subtract out the common RNAs leaving sequences expressed exclusively in the infected tissue. As described above, the identity of these sequences is determined by sequencing. Using the first procedure, it was shown that the gene *fadB* was being expressed *in vivo* (27). This gene product is involved in the degradation of fatty acids by β-oxidation. It was hypothesized that this enzyme is important in suppressing the inflammatory response, particularly responses mediated by arachidonic acid.

Evasion of the Host Immune Response - One of the major strategies used by bacteria when they cause disease is to evade the host's immune response. It is generally accepted that optimal efficacy of an antibiotic is dependent upon a fully functioning immune response system. Therefore, mechanisms that subvert this

process increase the organism's ability to survive at the site of infection. Similarly, methods that block immune evading processes should enhance the activity of the antibiotic and should aid the host in clearing the infection. Examples of microbial mechanisms of immune evasion are listed in Table 3 below:

Table 3. Mechanisms of Immune Evasion

Mechanism	Organism	Gene Product Involved
Walling off	*S. aureus*	Coagulase
Kill phagocytes	*S. pneumoniae*	Hemolysins
Acquire iron	*E. coli*	Siderophores
Evade phagocytosis	*S. aureus*	Protein A
Intracellular survival	*Salmonella*	Many genes
Resists products of oxidative burst	*S. aureus*	Catalase, superoxide dismutase

Virulence Factors, the Immune System and Antimicrobial Therapy - Recent literature reports have partially characterized certain antibacterials (Table 4) as possessing a wide variety of immune response modifier properties ranging from immunostimulatory to immunosuppressive (28-41). Although there are numerous contradictory and incomplete reports on this subject, there is an increasing database of antibacterials with immunomodulatory properties.

Table 4. Antimicrobial Effects on the Immune System

Drug(s)	Immunomodulatory Activity	Molecule(s) Examined and Results	References
ciprofloxacin	enhance T-cell function	Increase IL-2 in PHA stimulated PBLs;	28-30
		Increase IL-1 in macrophages	28
ciprofloxacin pefloxacin	enhance T-cell function	Increase IL-2 in human mononuclear leukocytes	31
ciprofloxacin amifloxacin	enhance T-cell function	Increase IL-2 in PHA stimulated PBLs	32
ceftazidime	enhance phagocytosis	Increase phagocytosis activity versus *S. aureus* and *P. aeruginosa* by human macrophages	33
ciprofloxacin	mitogen	Increase *de novo* pyrimidine biosynthesis	34,35
ciprofloxacin enoxacin pefloxacin norfloxacin	mitogen	Increase ^{3}H-thymidine uptake	35,36
ciprofloxacin	immunostimulation	Increase IL-2, IL-3, IL-4 ILN-γ, TNF-α, M-CSF, G-CSF	37
ceftazole	immunostimulation	Increase T-cell mitogenicity	38
teicoplanin	immunostimulation	Increase phagocytosis	39
cefmetazole	immunostimulation	Increase phagocytosis and ADCC of neutrophils	40
cefoxitin	immunostimulation	Increase phagocytosis and ADCC of neutrophils	41

The coordination of expression of immune response effector macromolecules (cytokines and adherence molecules) is essential for the actions of the immune and inflammatory responses to bacteria and bacterial virulence factors. Bacterial infections themselves can be immunomodulators. Antimicrobial agents may work

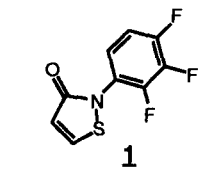
directly by mechanisms suggested in the table below, or indirectly by releasing enzymes and toxins from the bacterial cell through lysis, causing release of cytokines in addition to a transient inflammatory response. Antibacterials as positive immune response modulators may enhance potency of the antibacterial activity of these agents (42).

Modulation of Gene Regulation as an Anti-virulence Strategy - Recent reviews (43-45) have described the importance to bacteria of networks of intracellular signaling (see below for a partial listing) in the bacterium in response to its environment. The

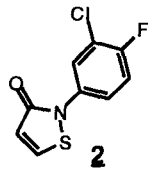
Table 5. Two-component Switch Controlled Virulence/Resistance Factors

Gene	Organism	Component	References
agr	*S. aureus*	global virulence regulator	46
*bvg*AS	*Bordetella pertussis*	virulence	47,48
*che*Y/*che*A	*S. typhimurium*	motility	49,50
pho	*S. typhimurium*	virulence	51,52
*van*RS	*Enterococcus faecalis*	van^R	53
kinA/*spo*0F	*Bacillus subtilis*	ability to sporulate	54
*alg*R/*agr*D	*P. aeruginosa*	alginate capsule	55-57
*omp*R/*Enz*	*Salmonella, E. coli Shigella*	virulence OMPs	58,59
pilG	*P. aeruginosa*	twitching motility	60
*tox*R	*Vibrio cholera*	virulence	61
*cpx*R/*cpx*A	*E. coli*	capsule	62
exo	*P. aeruginosa*	exoenzymes	63
fixL/*fix*J	*Rhizobium meliloti*	heme receptor	64
*sox*Q	*E. coli*	oxidative stress	65
pilS/*pil*R	*P. aeruginosa*	fimbriae	66
*fin*G/*fin*N	*S. typhimurium*	flagella	67
*rcs*B/*rcs*C	*E. coli*	capsule	68
*ntr*B/*ntr*C	enteric bacteria	nitrogen regulation	69,70
*omp*R/*omp*S	enteric bacteria	porins	71
*com*P/*com*A	*B. subtilis*	controls *srf*A	72
mry	group A streptococci	M protein virulence	73,74
*pmr*A	*S. typhimurium*	virulence	75
*prf*A	*Listeria monocytogenes*	virulence	76

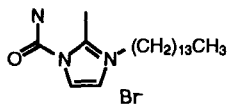
interruption of these signals may lead to the interruption of virulence and/or decrease in bacterial virulence factors. Such has been reported in seeking virulence-modifying agents against *P. aeruginosa* (57). Targeting a known two-component switch system in *P. aeruginosa*, the alginate biosynthetic pathway (AlgR2/AlgR1), these researchers screened for non-antibacterial, alginate-suppressing agents (57). Several inhibitors were identified as potent inhibitors of *alg*D promoter activity as shown below. Compounds **1** and **2** inhibited autophosphorylation of AlgR2, autophosphatase of AlgR2, and the phosphotransfer of the phosphate to AlgR1, but did not inhibit the binding of AlgR1 to the *alg*D promoter. Compounds **3** and **4** did not inhibit the autophosphorylation, autophosphatase or phosphotransferase of AlgR2, but did inhibit the binding of AlgR1 to the *alg*D promoter.



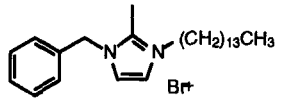
1



2



3



4

Other gene regulatory systems such as the N-acyl-homoserine lactones (77-79) and protein tyrosine phosphatases (80) exist in bacteria, and the tools of molecular biology will inevitably identify additional systems. Inhibition of any of these systems/pathways may generate novel agents capable of suppressing virulence. The similarity of many of these systems makes their inhibition an attractive target for pharmaceutical intervention.

References

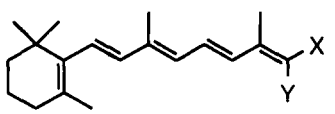
1. H. C. Neu, Science, 257, 1064 (1992)
2. B. Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons, NY (1988)
3. B. R. Glick, J.J. Pasternak. Molecular Biotechnology, ASM Press, Washington, DC (1994)
4. M.R.W. Brown, J.W. Costerton, P. Gilbert, J. Antimicrob. Chemother. 27, 565 (1991)
5. A.A. Salyers, D.D. Whitt. *Bacterial Pathogenesis*, ASM Press, Washington, DC (1994)
6. R.R. Iseberg, D.L. Voorkis, S. Falkow, Cell, 50, 769 (1987)
7. E.H. Beachey, J. Infect. Disease, 143, 325 (1981)
8. I. Ofek, W.A. Simpson, E.H. Beachey, J. Bact. 149, 426 (1982)
9. R.E. Isaacson, In: Virulence Mechanisms of Bacterial Pathogens, J.A. Roth (Ed.), ASM Press, pp. 28 (1988)
10. R.E. Isaacson, Avian Dis., 30, 28 (1985)
11. J.A. Roth, Virulence Mechanisms of Bacterial Pathogens, ASM Press (1988)
12. R.E. Isaacson (ed), Recombinant DNA Vaccines: Rationale and Strategy, Marcel Dekker, Inc. (1994)
13. S. Clegg, *In*: Recombinent DNA Vaccines, R.E. Isaacson (ed.), pp. 73-88 (1992)
14. A. Hostacka, L. Majtanova, Ceskoslovenska Epidemiologie, Micrbiologie, Immunologie 42(4), 197 (1993)
15. S.A. Doss, G. S. Tillotson, S.G. Amyes, J. Applied Bacteriol. 75(2), 123 (1993)
16. C.G. Gemmell, M. McLeod, Diag. Microbiol. Infect. Dis. 15(4 Suppl), 67S (1992)
17. Y. Hirakata, M. Kaku, R. Mizukane, K. Ishida, N. Furuya, T. Matsumoto, K. Tateda, K. Yamaguchi, Antimicrob. Agents Chemother. 36(9), 1922 (1992)
18. S.A. Sonstein, J.C. Burnham, Diag. Microbiol. Infect. Dis. 16(4), 277 (1993)
19. Y.X. Furet, J.C. Pechere, J. Antimicrob. Chemother. 26(Suppl B), 7 (1990)
20. S.M. Hammond, FEMS Microbiol. Letters 79(1-3), 293 (1992)
21. V.V. Tetz, L.L. Norman, J. Med. Microbiol. 41(4), 279 (1994)
22. P.D. Marsh, J. Dental Res. 71(7), 1431 (1992)
23. K.A. Homer, F. Manji, D. Beighton, J. Clin. Periodont. 19(5), 305 (1992)
24. K.A. Homer, F. Manji, D. Beighton, Arch. Oral Biol. 35(6), 421 (1990)
25. H. Cederlund, P.A. Mardh, J. Antimicrob. Chemother. 32, 355 (1993)
26. M.J. Mahan, J.M. Slauch, J.J. Mekelanos, Science, 259, 686(1993)
27. M.J. Mahan, J.W. Tobais, J.M. Slauch, P.C. Hanna, R.J. Collier, J.J. Mekelanos, PNAS USA , 92, 669 (1995)
28. K.G.E. Stunkel, G. Hewlett, H.J. Zeiler, Clin. Exp. Immunol., 86, 525 (1991)
29. D. Delfino, L. Borina, M.C. Berlinghieri, P. Mastroeni, Chemotherapia 4, 463 (1985)
30. K. Riesbeck, A. Forsgren, Eur. J. Clin. Microbiol. Infect Dis., 9, 409 (1990)
31. Y. Roche, M. Fay, M.-A. Gougerot-Pocidalo, Int. J. Immuopharmac., 10, 161 (1988)
32. K. Riesbeck, J. Anderson, M. Gullberg, A. Forsgren, Proc. Natl. Acad. Sci. USA., 86, 2809 (1989)
33. A.M. Cuffini, N.A. Carlone, L. Xerri, M.F. Pizzoglio, Journal of Antimicrobial Chemotherapy, 20, 261 (1987)
34. A. Forsgren, A. Bredberg, A.B. Pardee, S.F. Schlossman, T.F. Tedder, Antimicrobial Agents and Chemother., 31, 774 (1987)
35. A. Forsgren, S. Fand, Chemotherapy, 31, 768 (1987)

36. A. Forsgren, A. Bergh, M. Brandt, G. Hansson, Antimicrobial Agents and Chemotherapy., 29, 506 (1986)
37. K. Riesbeck, M. Sigvardsson, T. Leanderson, A. Forsgren, Journal of Immunology, 153,343 (1994)
38. K. Akahane, K. Furuhama, M. Kato, T. Une, T. Onodera, Chemotherapy, 36,300 (1990)
39. N.A. Carlone, A.M. Cuffini, M. Ferrero, V. Tullio, G. Avetta, Journal of Antimicrobial Chemother., 23, 849 (1989)
40. A.B. Rodriguez, C. Barriga, M. de la Fuente, Microbiology and Immunology, 35, 545 (1991)
41. A.B. Rodriguez, C. Barriga, M. de la Fuente, Comparative Immunology, Microbiology Infectious Diseases, 16, 37 (1993)
42. P. H. Lagrange, Pathologie Biologie, 41, 759 (1993)
43. J.B. Stock, A.J. Ninfa, A.M. Stock, Microbiol. Rev. 53, 450 (1989)
44. J.S. Parkinson, Cell 73, 857 (1993)
45. F.D. Russo, T.J. Silhavy, Trends Microbiol. 1, 306 (1993)
46. A. Abdelnour, S. Arvidson, T. Bremell, C. Ryden, A. Tarkowski, Infect Immun, 61, 3879 (1993)
47. J.G. Coote, , J. Gen. Microbiol., 137, 2493 (1991)
48. B.J. Akerley, J.F. Miller, , J. Bacteriol., 175, 3468 (1993)
49. K. Volz, Biochemistry, 32, 11741 (1993)
50. R.B. Bourret, J.F. Hess, M.I. Simon, Proc. Natl. Acad. Sci. U. S. A., 87, 41 (1990)
51. R.B. Finlay, F. Heffron, S. Falkow, Science, 243, 940 (1989)
52. S.I. Miller, Mol Microbiol, 5, 2073 (1991)
53. M. Arthur, C. Molinas, P. Courvalin, J. Bacteriol., 174 2582 (1992)
54. D. Burbulys, K.A. Trach, J.A. Hoch, Cell, 64, 545 (1991)
55. V. Deretic, C.D. Mohr, D.W. Martin, Mol. Microbiol., 5, 1577 (1991)
56. J. B. Goldberg, T. Dahnke, Mol. Microbiol., 6, 59 (1992)
57. S. Roychoudhury, N.A. Zielinski, A.J. Ninta, N.E. Allen, L..N. Junbgheim, T.I. Nicas, A.M. Chakrabarty, Proc. Natl. Acad. Sci. USA 90, 965 (1993)
58. M.L. Bernardini, A. Fontaine, P.J. Sansonetti, J. Bacteriol., 172 6274 (1990)
59. J. Waukau, S. Forst, J. Bacteriol., 174, 1522 (1992)
60. A. Darzins, J. Bacteriol., 175, 5934 (1993)
61. V. DiRita, J. Mol. Microbiol., 6, 451 (1992)
62. J. Dong, S. Iuchi, H. S. Kwan, Z. Lu, E.C.C. Lin, Gene, 136, 227 (1993)
63. D. W. Frank, B. H. Iglewski, J Bacteriol, 173, 6460 (1991)
64. M.A. Gilles-Gonzalez, G. Gonzalez, J Biol Chem., 268, 16293 (1993)
65. J.T. Greenberg, J.H.Chou, P.A. Monach, B. Demple, J. Bacteriol., 173, 4433 (1991)
66. M. Hobbs, E.S.R. Collie, P.D. Free, S.P. Livingston, J.S. Mattick, Mol. Microbiol., 7, 669 (1993)
67. V.M. Irikura, M. Kihara, S. Yamaguchi, H. Sockett, R.M. McNab, J. Bacteriol., 175, 802 (1993)
68. P. Jayaratne,W.J. Keenleyside, P.R. Maclachlan, C. Dodgson, C. Whitfield, J Bacteriol, 175, 5384 (1993)
69. J. Keener, S. Kustu, Proc. Natl. Acad. Sci. U. S. A., 85, 4976 (1988)
70. B.T. Nixon, C.W.Ronson, F.M. Ausubel, Proc. Natl. Acad. Sci. U. S. A., 83, 7850 (1986)
71. K. Nakashima, K. Kanamaru, H. Aiba, T. Mizuno., FEMS Microbiol Lett, 82, 43 (1991)
72. M.M. Nakano, L.A. Xia, P. Zuber, J. Bacteriol, 173, 5487 (1991)
73. A.F. Lois, M. Weinstein, G.S. Ditta, D.R. Helinski, J. Biol. Chem., 268, 4370 (1993)
74. J. Perez-Casal, M.J. Caparon, J.R. Scott, J. Bacteriol., 173, 2617 (1991)
75. K.L. Roland, L.E. Martin, C.R. Esther, J.K. Spitznagel, J. Bacteriol., 175, 4154 (1993)
76. K. Wernars, K. Heuvelman, S. Notermans, E. Domann, M. Leimeisterwachter, T. Chakraborty, Appl. Environ. Microbiol., 58, 765 (1992)
77. S. Swift, N.J. Bainton, M.K. Winson, Trends in Microbiol., 2(5), 193, (1994)
78. N.J. Bainton, B.W. Bycroft, S.R. Chhabra, P. Stead, L. Gledhill, P.J. Hill, C.E.D. Rees, M.K. Winson, G.P.C. Salmond, G.S.A.B. Stewart, P. Williams, Gene 116, 87 (1992)
79. P. Williams, N.J. Bainton, S. Swift, S.R. Chhabra, M.K. Winson, G.S.A.B. Stewart, G.P.C. Salmond, B.W. Bycroft, FEMS Microbiol. Letters 100, 161 (1992)
80. K.L. Guan, J.E. Dixon, Seminars in Cell Biology 4, 389 (1993)

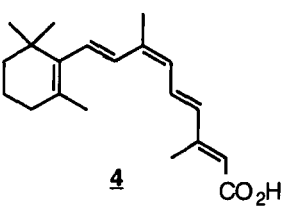
Chapter 13. Retinoids for the Treatment of Oncological Diseases

Alex M. Nadzan
Ligand Pharmaceuticals, Inc., San Diego, CA 92121

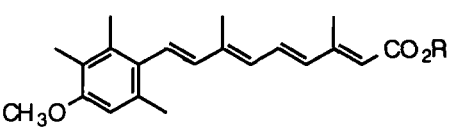
Introduction - Retinoids consist of a family of naturally occurring compounds including vitamin A (retinol, **1**), retinal (**2**), all-*trans* retinoic acid (ATRA, **3**), 9-*cis* retinoic acid (9-*cis* RA, **4**), and 13-*cis* retinoic acid (13-*cis* RA, **5**), as well as a large number of synthetic derivatives, of which etretinate (**6**), acitretin (**7**), and fenretinide (4-HPR, **8**) serve as representative examples (1-7). Retinoids exert profound effects on many important cellular processes including development, vision, reproduction, bone formation, hematopoiesis, metabolism, immune function, cellular proliferation and differentiation, and cell death (8). Several excellent comprehensive monographs have reviewed recently the biological actions and chemistry of this class of compounds (9-11). The potent actions of retinoids on cellular proliferation and differentiation has stimulated extensive research on the potential of these agents for the treatment of proliferative diseases such as acne, psoriasis, and numerous cancers (7,12-14). Although recent reviews in this series have focused on the use of retinoids in dermatological disease states (15,16), none have dealt with their potential for the treatment and prevention of oncological diseases. The present chapter will highlight recent advances in molecular and cellular biology of retinoid receptors and retinoids that have lead to intense interest in these regulators of gene transcription, and summarize the most current evidence supporting the role for retinoids in cancer chemotherapy. In addition, the recent developments toward new, receptor selective retinoids that may display improved activity/toxicity profiles and prove efficacious either as single agents or in combination therapy with growth factors, cytotoxic agents, or biological response modifiers will be reviewed.



	X	Y
1:	CH_2OH	H
2:	CHO	H
3:	CO_2H	H
5:	H	CO_2H
8:	CONH–⟨⟩–OH	H
9:	CONH–⟨⟩–CO_2H	H



4



6: R = CH_2CH_3

7: R = H

Background - Vitamin A or retinol is obtained exclusively from the diet in the form of retinol, retinyl esters or carotenoids (β-carotene, pro-vitamin A), and is stored primarily in the liver in the form of retinyl esters (17). Vitamin A is transformed in the body to a variety of active metabolites including retinal, ATRA, 9-*cis* RA, and 13-*cis* RA. Although retinal is required for visual function, ATRA is essential for growth and differentiation of epithelial tissue and for reproduction, embryonic development, and growth of bone.

Copyright © 1998 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Numerous synthetic retinoids have been developed for use in treating a variety of proliferative disorders (7,13). Chemically synthesized natural retinoids ATRA (Retin A™) and 13-*cis* RA (Accutane™) are marketed for the treatment of severe acne, while synthetic etretinate (Tegison™) is prescribed for severe, refractory psoriasis. More recently, ATRA, 13-*cis* RA, and etretinate have shown promise in the control of certain cancers (7,14). Widespread clinical use of these agents, particularly as chemopreventives, is severely limited due to the associated undesirable side effects which accompany treatment. These effects include skin irritation, headache, mucocutaneous, lipid and bone toxicity, and profound teratogenicity (18). Since it is likely that both beneficial and detrimental effects of retinoids arise through activation of multiple retinoid receptor subtypes, development of subtype selective retinoids may afford effective agents with improved therapeutic indices.

Retinoid Receptors - Retinoid receptors (RRs) are members of a large super-family of nuclear, intracellular receptors (IRs) that include the steroid, thyroid, and vitamin D hormone receptors, as well as several "orphan" receptors for which no known endogenous ligands have been identified (19). The modular nature of IRs permits exchange of receptor domains, including the DNA and ligand binding domains, to afford chimeric receptors with predictable hybrid properties. Such chimeras have been utilized to develop powerful cell-based "*cis/trans*" or cotransfection assays that have greatly advanced our knowledge of the IR family. Recent reviews and papers have described the discovery of the IR family (8,19,20), the mechanisms of nuclear hormone action (21-23), and the application of IR technology to the discovery of novel compounds acting at these receptors (24,25).

Molecular and cellular biological studies have shown that retinoids elicit many of their actions by binding to and activating RRs. These receptors function as ligand-inducible transcription factors that regulate gene expression by binding to specific hormone response elements (HREs) in the promoter regions of genes to either activate or repress transcription (8,19). A total of six RRs have been identified to date, comprising two distinct subclasses. Retinoic acid receptors (RARs) consist of three highly homologous proteins, RARα, RARβ and RARγ, that are activated directly by ATRA and 9-*cis* RA, both of which bind with high affinity to these receptors (3, 26). The second subclass, retinoid X receptors (RXRs), also contains three highly homologous proteins, RXRα, RXRβ, and RXRγ. 9-*cis* RA binds and activates RXRs directly, while ATRA does not bind with high affinity to RXRs but activates indirectly via metabolic conversion *in vitro* or *in vivo* to 9-*cis* RA (3,4). Importantly, vitamin A and retinal bind poorly to both RR subclasses, and thus each must undergo bioconversion to ATRA or 9-*cis* RA to activate these receptors (17). Since 9-*cis* RA binds and activates both classes of RRs, it likely plays a key role in regulating retinoid-responsive pathways. RARs are distinct genetically from RXRs, but are highly homologous to one another and display amino acid sequence homologies of >97% and >76% for their respective DNA binding domains (DBDs) and ligand binding domains (LBDs). RXRs also exhibit high sequence homologies with one another, *i.e.*, >92% and >86% for the DBDs and LBDs, respectively (8). Although X-ray structural data are available for the DBDs of several of the IRs, no tertiary structural information has been published to date for the LBDs or the intact receptors of any IRs (27).

RRs bind to specific HREs on DNA as dimeric pairs. RARs bind solely as heterodimers with RXRs, while RXRs can bind either as homodimers or as heterodimers with other members of the IR family, including thyroid hormone, vitamin D, and peroxisome proliferator-activated receptors (8). Diversity in hormonal signaling is thus achieved through the nature of the dimer and ligand, the specific HREs under control of the ligand-receptor complex, and the RR subtypes present in the tissue (8, 28). These complex signaling pathways are the basis of many of the diverse biological effects elicited by the retinoids and their interactions with hormones and other biomolecules.

RETINOIDS AND CANCER

Recent reviews have summarized the compelling evidence supporting the ability of retinoids to inhibit the process of carcinogenesis in a variety of cell types (7,29-31), in experimental animal models (32), and in humans (14,33,34). Such investigations have utilized retinoids both in chemotherapeutic or chemopreventive modes either as single agents or in combination with growth factors, cytokines, cytotoxic agents, and biological response modifiers. Moreover, epidemiological studies have indicated an inverse relationship between the dietary consumption of β-carotene (pro-vitamin A) and the development of certain human cancers such as lung, esophageal, urinary tract, breast, and carcinoma of the cervix (14). In human cancer prevention studies, retinoids have shown promise for skin diseases such as actinic keratosis, keratoacanthoma, and basal cell carcinoma, and for nondermatologic premalignant disorders including oral leukoplakia, cervical displasia, bronchial metaplasia, laryngeal papillomatosis, and myelodysplastic syndromes (29). Most recently, retinoids have demonstrated success in the treatment of certain refractory malignancies such as advanced cutaneous squamous and basal cell cancers, mycosis fungoides, and acute promyelocytic leukemia as detailed below.

Mode of Action - The underlying mechanisms of retinoid-based suppression of carcinogenesis reside in the ability of these agents to regulate cellular growth and induce differentiation in a wide variety of pre-neoplastic and neoplastic cell types (30), and to induce apoptosis (programmed cell death) in certain cells (35,36). These effects result from the actions of vitamin A metabolites, ATRA and 9-*cis* RA, and synthetic retinoids on RRs which regulate the expression of specific genes and, hence the synthesis of mRNAs and many important cellular proteins that play pivotal roles in the growth, differentiation, and viability of cells. These cellular proteins encompass a large number of growth factors and cytokines, several growth-related hormones, a variety of intracellular and secreted enzymes, extracellular matrix proteins and their related proteases and inhibitors, human viruses such as papillomavirus (HPV), cytomegalovirus (HCMV), and immunodeficiency virus (HIV)), as well as several oncogenes including *c-fos* and *N-myc*. The regulatory actions on these proteins are highly complex and can occur at the transcriptional, translational or post-translational levels as summarized in a recent review (30). For example, retinoids inhibit the expression of oncogenes *c-fos* and *N-myc* at the transcriptional stage, however ATRA suppresses the actions of AP-1, the complex of protooncoproteins *jun* and *fos*, at the protein level through the interaction of RARs directly with the AP-1 complex (37).

Of particular relevance to cancer are the actions of retinoids on the enzymes ornithine decarboxylase (ODC) and tissue transglutaminase (Tgase). Retinoids inhibit phorbol ester induced expression of ODC in mouse epidermis which has served as an assay to assess the antitumor potential of retinoids in skin disease (38). Recent studies have shown that ATRA suppresses ODC mRNA as well as end product putrescene levels, but it is not certain whether this effect is transcriptional or post-transcriptional (30). Tgases are a group of calcium-dependent matrix cross-linking enzymes also regulated by retinoids. Tissue Tgase (type II) is a soluble protein which is highly expressed in myeloid cells and likely plays a role in regulating cell growth and cell death (39,40). Experiments have demonstrated that retinoids regulate tissue Tgase in these cells, and compounds selective for the RXR receptor subtypes appear to be potent inducers of this enzyme (41). Regulation of growth factors by retinoids also has important implications in cancer chemotherapy and chemoprevention in view of studies demonstrating additive or synergistic effects for combinations of retinoids such as ATRA, 13-*cis* RA, and 9-*cis* RA with interferons (IFN-α, - β, and - γ) on the inhibition of growth in leukemia and carcinoma cell lines, and in experimental animals (30,32,42). Such combination regimens have produced promising clinical results in the treatment of advanced human malignancies as highlighted below.

Finally, additional actions of direct relevance to carcinogenesis include the ability of retinoids to inhibit the adhesive and invasive properties of tumor cells, which are linked to the suppressive actions on the synthesis of enzymes that degrade basement membranes and extracellular matrix (30), and the ability to curb angiogenesis in a chick embryo model and in animals (42,43).

Human Clinical Trials - Until recently, the majority of trials have been limited to studies with essentially four retinoids: ATRA, 13-*cis* RA, etretinate, and fenretinide. High response rates were observed for patients with acute promyelocytic leukemia (APL), a disorder characterized by a specific chromosomal translocation defect between chromosomes 15 and 17, involving the expression of RARα (44). APL patients with this translocation experience 50-94% remission rates when treated with ATRA, but not with 13-*cis* RA. Unfortunately, remission is relatively brief (1-14 mos.) apparently due to a precipitous drop in serum ATRA levels upon repeated exposure. 9-*cis* RA may be a viable alternative to ATRA for APL in view of its maintained serum drug levels in man with chronic treatment (45,46). Use of retinoid therapy for other hematologic malignancies such as myelodysplastic syndrome, chronic myelogenous leukemia, and cutaneous T-cell lymphoma have not been successful, although positive results were achieved with mycosis fungoides patients treated with a combination of etretinate and bleomycin, cyclophosphamide, and prednisone (47). Retinoid therapy also has been successful in treating two types of epithelial tumors. Patients with squamous-cell carcinoma of the skin were treated with 13-*cis* RA either as a single agent or in combination with IFN-α to give response rates of 55-60% (48). Significantly, patients with cervical cancer (49) or advanced renal adenocarcinoma (50) also responded to combination therapy with 13-*cis* RA and IFN-α.

In chemopreventive trials, 13-*cis* RA demonstrated significant activity in reversing oral leukoplakia, a common premalignant lesion of the aerodigestive tract (51). Favorable responses were observed at both high and low doses of 13-*cis* RA, and similar results were obtained both for ATRA and etretinate (14). In other premalignancies, topical ATRA and systemic etretinate proved effective for actinic keratosis of the skin, producing response rates of 50 and 95%, respectively (14). In addition, topical ATRA when dispensed in a cervical cap to patients with early stage cervical intraepithelial neoplasia (CIN II) gave a 33% complete response rate (52). Significantly, suppression of secondary primary tumors in patients with head and neck squamous-cell carcinoma also was achieved employing high dose 13-*cis* RA (53,54).

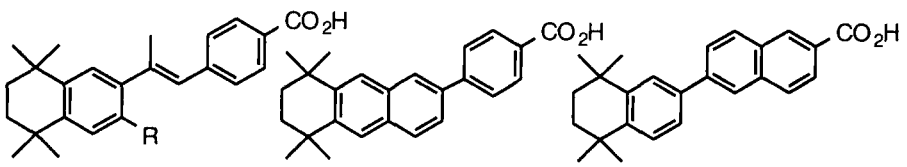
Although the results of the above chemopreventive studies are quite encouraging, relapse rates were high in most studies upon cessation of drug treatment (14). Moreover, noncompliance also was high due to the occurrence of multiple adverse side effects, particularly at higher doses of retinoids and in the combination studies with IFN-α. Of the retinoids available for clinical study, fenretinide appears to produce the least number of adverse side effects, and thus it is in trials as a chemopreventive agent for breast cancer and for oral leukoplakia (55). Although the results of the breast cancer study are not available yet, preliminary results of the oral leukoplakia trial are encouraging (56). Similar promising results were reported for patients with oral leukoplakia and cervical dysplasia in trials conducted with 4-HCR (**9**), a closely related analogue of fenretinide (57). Interest in fenretinide for other chemopreventive trials has been prompted by recent reports of successful cellular and *in vivo* animal experiments in preventing superficial bladder cancer (32), prostate cancer (58), and for breast and skin cancer in combination with tamoxifen and interferons, respectively (55).

RECEPTOR SELECTIVE RETINOIDS

The discovery of retinoids with improved therapeutic indices and reduced toxicity has been a major objective of retinoid research over the last ten to fifteen years (7). Many compounds have been evaluated for the ability to inhibit growth and

induce cellular differentiation in a variety of cell lines, and inhibit tumor growth in animal models of cancer. Only very recently has it been possible to establish the receptor profiles of such compounds, thus this discussion will focus primarily on retinoids that have been characterized for the ability to bind, activate, or inhibit RR subtypes.

RAR Selective Agonists - The availability of cloned RRs and the appropriate cell-based assays to measure gene activation has prompted the use of binding and cotransfection assays to characterize the RR profiles of known synthetic retinoids. The aims of these studies were to gain insight into receptor selectivity and utilize such information to rationalize biological activity data and design new subtype selective retinoids (59-61). These investigations have revealed that highly potent retinoids such as TTNPB (**10**), TTAB (**12**), and TTNN (**13**) activate only the RAR subtypes, and show either no selectivity for the individual RAR isoforms, in the case of TTAB, or marginal selectivity (5- to 10-fold) for TTNPB and TTNN at RARβ and RARγ vs. RARα (59). AM 80 (**14**) and AM 580 (**15**), two potent amide-based retinoids, exhibit selectivity for RARα and RARβ (20- to 100-fold), and RARα isoforms (69-fold), respectively (61,62). The degree of selectivity is somewhat dependent on the cotransfection assay and reporter system used, as others have reported only modest selectivity for AM-580 at RARα relative to RARβ (59). Several other synthetic retinoids appear to activate both RARβ and RARγ in preference RARα [59,61]. These include CD 495 (**16**), CD 564 (**17**), and TTNN (**13**). Such RAR subtype selectivity is understandable in view of the greater sequence homology in the LBDs of RARβ and RARγ relative to the RARα isoform (8).

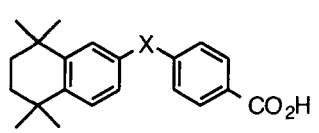


10: R = H

11: R = CH_3

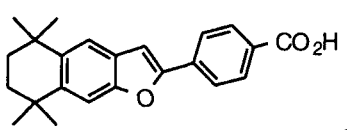
12

13

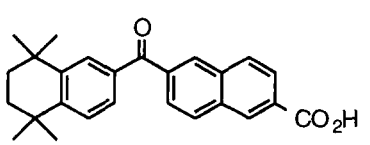


14: X = NH-CO

15: X = CO-NH



16

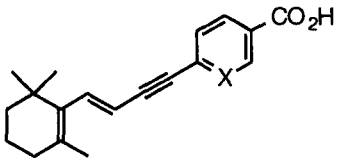


17

Two novel series of acetylenic retinoids, compounds **18**-**19** and **20**-**25**, were designed by introducing conformational constraints into ATRA and TTNPB, respectively (63). Several members of the series, including compounds **18**-**21**, demonstrated high potency (EC_{50} values of 0.3-15 nM) and selectivity (>100-fold) for RARβ and RARγ subtypes over RARα. These compounds also were reported to be potent inhibitors of ODC in the hairless mouse assay. Select heterocyclic analogues, **22**-**24**, showed some preference for the RARβ subtype. Although most of the above compounds failed to activate RARα in the cotransfection assay, many bound with reasonable affinity to this subtype. Significantly, these compounds inhibited AP-1 activity in cells transfected with RARα, thus demonstrating the ability to separate inhibition of AP-1 and the transactivation process. Such compounds may prove to be effective agents with less retinoid-induced toxicity, since some of the toxic effects appear to be associated with transactivation of RARα (64). The ethyl ester of **23**,

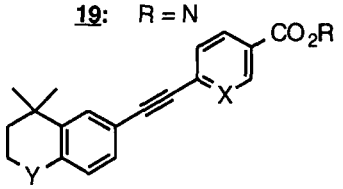
AGN190,168 (tazarotene, **25**), is currently in Phase III clinical trials for the topical treatment of acne and psoriasis. Tazarotene is effective in psoriasis with significantly less irritation than ATRA or 13-*cis* RA (65).

Finally, CD 437 (**26**), CD 1530 (**28**), and CD 666 (**29**) demonstrate selectivity for the RARγ subtype (66). Interestingly, CD 271 (Adapalene, **27**), the corresponding methyl ether of **26**, displays RARβ selectivity in binding (67). The RARγ selectivity of **28** and **29**, coupled with the difference in selectivity between **26** and **27**, suggests that RARγ activity can be enhanced by incorporation of a hydroxyl function into the molecule. It remains to be determined if this is a general phenomenon. Adapalene is in clinical trials as a topical anti-acne agent (68).

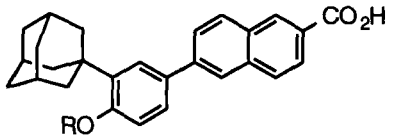


18: R = CH

19: R = N

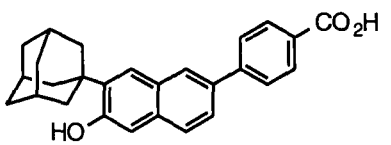


	X	Y	R
20:	CH	$C(CH_3)_2$	H
21:	N	$C(CH_3)_2$	H
22:	CH	S	H
23:	N	S	H
24:	N	O	H
25:	N	S	CH_2CH_3

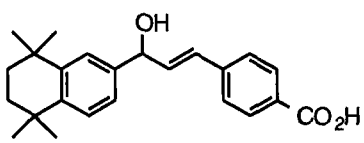


26: R = H

27: R = CH_3



28

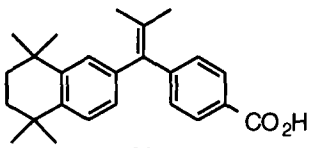


29

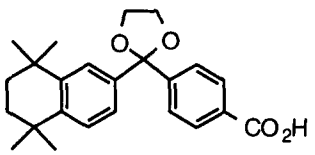
RXR Selective Agonists - Within the last three years, novel classes of RXR-selective retinoids have emerged. SR11217 (**30**) and SR11237 (**31**) were derived from evaluating a series of retinoids for activity with cotransfected RXRα (69). Neither compound exhibited activity at 10^{-8} M, however, both displayed RXRα activity comparable to 9-*cis* RA at 10^{-7} M. Compounds **30** and **31** were inactive at RAR subtypes. Cotransfection studies also revealed that these compounds activate RXR homodimers but not RAR-RXR heterodimers, demonstrating the activation of RXR-dependent response pathways in preference to RAR-dependent pathways.

A second, more potent series of RXR selective retinoids was designed by capitalizing on the initial observation that introduction of a 3-methyl substituent to RAR selective TTNPB (**10**) afforded 3-MeTTNPB (**11**), having modest activity at all six RR subtypes (70). Similarly, incorporation of 3-methyl substituents to weakly active **32** and **33** resulted in two series of RXRα,β,γ selective compounds, exemplified by **34** and **35**. The most potent compound, LGD1069 (**35**), activated RXR subtypes in the 20-40 nM range with 50- to 70-fold selectivity for RXRs over RARs (71). Further

refinements in the SAR of this series produced analogues of comparable or improved potency, with enhanced selectivity for RXR subtypes (72). These included the bridgehead epoxide (**36**), cyclopropane (**37**), and 3-pyridinecarboxylic acid (**38**) derivatives. LGD1069, currently in clinical trials for the treatment of cancer, was reported to induce tissue Tgase in HL60 cells and apoptosis when co-administered with the RAR selective TTNPB (72, 73).

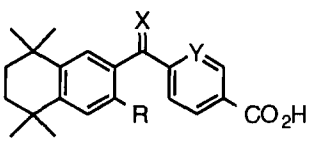


30

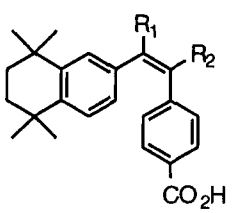


31

Molecular modeling is being utilized to gain insight into conformational and structural features that impart receptor subtype selectivity. Two approaches are being taken, the first of which involves careful comparison of closely related members within a series, e.g., RXR-selective **35** and nonselective **33** (71). The second approach entails comparison of constrained RAR-selective analogues (**10** and **12**), RXR-selective compounds (**30** and **31**), or RXR-active analogues (**39**-**41**), with low energy conformations of 9-*cis* RA (59,74). These investigations should aid in the development of future generations of selective agents.



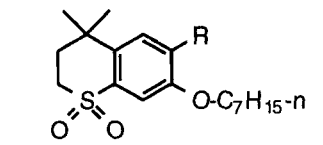
	R	X	Y
32:	H	O	CH
33:	H	CH_2	CH
34:	CH_3	O	CH
35:	CH_3	CH_2	CH
36:	CH_3	CH_2O	CH
37:	CH_3	CH_2CH_2	CH
38:	CH_3	CH_2	N

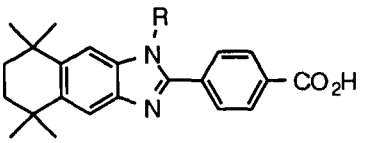


	R_1	R_2
39:	CH_3	H
40:	H	CH_3
41:		

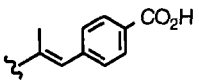
Retinoid Antagonists - In addition to the above novel agonists, several RAR-selective antagonists have been reported recently (75,76). A series of related sulfone derivatives, Ro 41-5253 (**42**), Ro 46-5471 (**43**), and Ro 46-8515 (**44**), exhibited selectivity for RARα in binding assays, and in a RARα transactivation assay, antagonized the actions of ATRA and other synthetic retinoids in a dose dependent manner. Ro 14-5253 also antagonized the effects of ATRA on HL-60 cell differentiation and on B-lymphocyte polyclonal activation. Most significantly, Ro 41-5253 inhibited the teratogenic effects of RARα selective, AM-580 in rat limb bud cell cultures and in mice, suggesting a significant role for RARα in the teratogenic effects of retinoids (64). Other RAR antagonists reported include TD550 (**45**), TD650 (**46**), BIPh (**47**), BIBn (**48**), and benzodiazepine derivative **49**; all of these compounds inhibited retinoid-induced differentiation of human promyelocytic leukemia HL60 cells (77-79). SR11335 (**50**) represents an unusual antagonist that blocks ATRA and 9-*cis* RA induced activation of HIV-1 RAR response element (80). The selectivity profile of **50** was not disclosed. As yet, no RXR-selective antagonists have been identified,

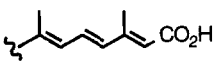
although potential lead compounds in the LGD1069 series were reported to bind, but not transactivate RXR subtypes (71).

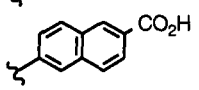


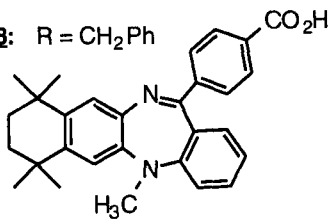


47: R = Ph

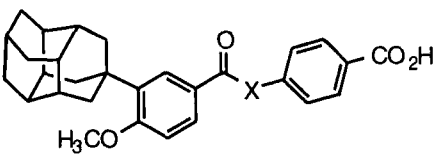
42: R = 

43: R = 

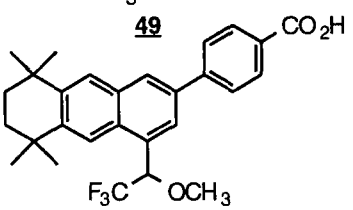
44: R = 

48: R = CH_2Ph 

49



45: X = NH **46**: X = O



50

SUMMARY AND FUTURE DIRECTIONS

Retinoid agonists and antagonists with selectivity for individual RR subtypes will undoubtedly provide valuable tools to elucidate the physiological and pharmacological actions regulated by these receptors. Although many of the known retinoids activate RARs, several new generation compounds activate RXRs including RXR selective, LGD1069 and SR11217, and 9-*cis* RA which activates both classes of RRs. These agents are beginning to define the roles of RXRs in retinoid biology. Cellular studies with LGD1069 and 9-*cis* RA have linked RXRs to the induction of apoptosis in certain cell types, which may have important implications in cancer chemotherapy (72,81). Recent studies also show that LGD1069 and SR11217 do not by themselves affect cellular proliferation and differentiation, but potentiate the actions of RAR selective retinoids (72,82). Future biological investigations with subtype selective compounds and ongoing clinical trials with 9-*cis* RA and LGD1069 will ultimately determine the potential for such agents in cancer chemotherapy (46,73).

References

1. D.S. Goodman and W.S. Blaner in "The Retinoids," Vol. 2, M.B. Sporn, A.B. Roberts and D.S. Goodman, Eds., Academic Press, Orlando, FL, 1984, p.1.
2. M.E. Cullen and M.H. Zile, J.Biol.Chem., 260, 10590 (1985).
3. R.A. Heyman, D.J. Mangelsdorf, J.A. Dyck, R.B. Stein, G. Eichele,R.M. Evans and C. Thaller, Cell, 68, 397 (1992).
4. A.A. Levin, L.J. Sturzenbecker, S. Kazmer, T. Busukowski, C. Huselton, G. Allenby, J. Speck, C.L. Kratzeisen, M. Rosenberger, A. Lovey and J.F. Grippo, Nature, 355, 359 (1992).
5. W. Bollag in "Retinoids: New Trends in Research and Therapy," J.H. Saurat, Ed., Karger, Basel, 1985, p. 274.
6. P. Loeliger, W. Bollag and H. Mayer, Eur.J.Med.Chem., 15, 9 (1980).
7. Y.F. Shealy, Prev.Med., 18, 624 (1989).
8. D.J. Mangelsdorf, K. Umesono and R.M. Evans in "The Retinoids," M.B. Sporn, A.B. Roberts and D.S. Goodman, Eds., Raven Press, New York, NY, 1994, pp. 319.
9. "Chemistry and Biology of Synthetic Retinoids," M.I. Dawson and W.H. Okamura, Eds., CRC Press, Boca Raton, FL, 1990.

10. "Retinoids: Progress in Research and Clinical Applications," M.A. Livrea and L. Packer, Eds., Marcel Dekker, New York, N.Y., 1993.
11. "The Retinoids: Biology, Chemistry and Medicine," 2nd Edition, M.B. Sporn, A.B. Roberts and D.S. Goodman, Eds., Raven Press, New York, N.Y., 1994.
12. M. Verschoore, M. Bouclier, J. Czernielewski and C. Hensby, Dermatol.Clin., 11, 107 (1993).
13. G.L. Peck and J.J. DiGiovanna in "The Retinoids: Biology, Chemistry and Medicine," 2nd Edition, M.B. Sporn, A.B. Roberts and D.S. Goodman, Ed., Raven Press, New York, N.Y., 1994, p. 631.
14. W.K. Hong and L.M. Itri in "The Retinoids: Biology, Chemistry and Medicine," 2nd Edition, M.B. Sporn, A.B. Roberts and D.S. Goodman, Ed., Raven Press, New York, N.Y., 1994, p. 597.
15. M.C. Venuti, Ann.Rept.Med.Chem., 22, 201 (1987).
16. E.L. Smith and J.J. Tegeler, Ann.Rept.Med.Chem., 24, 177 (1989).
17. W.S. Blaner and J.A. Olson in The Retinoids: Biology, Chemistry and Medicine," 2nd Edition, M.B. Sporn, A.B. Roberts and D.S. Goodman, Eds., Raven Press, New York, N.Y., 1994, p. 229.
18. R.B. Armstrong, K.O. Ashenfelter, C. Eckhoff, A.A. Levin and S.S. Shapiro in "The Retinoids: Biology, Chemistry and Medicine," 2nd Edition, M.B. Sporn, A.B. Roberts and D.S. Goodman, Ed., Raven Press, New York, N.Y., 1994, p. 545.
19. R. Evans, Science, 240, 889 (1988).
20. S. Green and P. Chambon, Trends.Genet., 4, 309 (1988).
21. M-J Tsai and B.W. O'Malley, Annu.Rev.Biochem., 63, 451 (1994).
22. D.P. McDonnell, B. Clevenger, S. Dana, D. Santiso-Mere, M.T. Tzukerman and M.A.G. Gleeson, J.Clin.Pharmcol., 33, 1165 (1993).
23. D.F. Smith and D.O. Toft, Mol.Endo, 7, 4 (1993).
24. T.S. Berger, Z. Parandoosh, B.W. Perry and R.B. Stein, J.Ster.Biochem.Molec.Biol., 41, 733 (1992).
25. R.B. Stein in "New Drugs from Natural Resources," J. Combes, Ed., IBC Technical Services, Ltd., U.K., 1992, p. 13.
26. E.A. Allegretto, M.R. McClurg, S.B. Lazarchik, D.L.Clemm, S.A. Kerner, M.G. Elgort, M.F. Boehm, S.K. White, J.W. Pike and R.A. Heyman, J.Biol.Chem., 268, 26625 (1993).
27. L.P. Freedman and B.F. Luisi, J.Cellular Biochem., 51, 140 (1993).
28. M. Leid, P. Kastner and P. Chambon, TIBS, 17, 427 (1992).
29. S.M. Lippman, J.F. Kessler and F.L. Meyskens, Jr., Canc.Treat.Rep., 71, 391 (1987).
30. L.J. Gudas, M.B. Sporn and A.B. Roberts in "The Retinoids: Biology, Chemistry and Medicine," 2nd Edition, M.B. Sporn, A.B. Roberts and D.S. Goodman, Ed., Raven Press, New York, N.Y., 1994, p. 443.
31. R. Lotan, Canc.Res.(Supp.), 54, 1987s (1994).
32. R.C. Moon, R.G. Mehta and K.V.N. Rao in "The Retinoids: Biology, Chemistry and Medicine," 2nd Edition, M.B. Sporn, A.B. Roberts and D.S. Goodman, Ed., Raven Press, New York, N.Y., 1994, p.573.
33. M.S. Tallman an P.H. Wiernik, J.Clin.Pharmacol., 32, 868 (1992).
34. S.M. Lippman, S.E. Benner and W.K. Hong, J.Clin. Oncol., 12, 851 (1994).
35. R.A. Schwartzman and J.A. Cidlowski, Endocrine Rev., 14, 133 (1993).
36. Y. Yang, M.S. Vacchio and J.D. Ashwell, Proc.Natl.Acad.Sci., 90, 6170 (1993).
37. H-F. Yang-Yen, X-k. Zhang, G. Graupner, M. Tzukerman, B. Sakamoto, M. Karin and M. Pfahl, The New Biologist, 3, 1206 (1991).
38. M.I Dawson, W-R Chao, P.D. Hobbs and T. Delair in "Chemistry and Biology of Synthetic Retinoids", M.I. Dawson and W.H. Okamura, Eds., CRC Press, Boca Raton, FL, 1990, p. 385.
39. E.A. Chiocca, P.J. Davies and J.P. Stein, J.Cell.Biochem., 39, 293 (1989).
40. P.J.A. Davies, M.P. Martaugh, W.T. Moore, G.S. Johnson and D.J. Lucas, J.Biol. Chem., 260, 5166 (1985).
41. L. Nagy, V.A. Thomazy, G.L. Shipley, L. Fesus, W. Lamph, R.A. Heyman, R.A.S. Chandraratna and P.J.A. Davies, Molec.Cell.Bio., 15, in press (1995).
42. W. Bollag, S. Majewski and S. Jablonska, Leukemia, 8, 1453 (1994).
43. T. Oikawa, I. Okayasu, H. Ashino, I. Morita, S-i. Murota and K. Shudo, Eur.J.Pharmacol., 249, 113 (1993).
44. A. Agadir, M. Cornic, B. Gourmel, P. Lefebvre, L. Degos and C.Chomienne, Cell. and Mol. Bio., 40, 263 (1994).
45. R.P. Warrell, Jr., Blood, 82, 1947 (1993).
46. F. Benedetti, V. Miller, J. Rigas, W. Tong, E. Ulm, J. Truglia, G. Gill and R.P. Warrel, Jr., Proc.Annu.Meet.Am.Assoc.Canc.Res., 36, A1283 (1995).
47. H. Zachariae and K. Thestrup-Pedersen, Acta.Derm.Venereol. (Stockh), 67, 433(1987).
48. S.M. Lippman, D.R. Parkinson, L.M. Itri, et al., J.Natl.Canc.Inst., 84, 235 (1992).
49. S. Lippman, J. Kavanagh, M. Paredes-Espinoza, et al., J.Natl.Canc.Inst., 84, 241 (1992).
50. R.J. Motzer, T. Murray-Law, L. Schwartz, P. Fischer, H.I. Scher and D.M. Nanus, Proc.Am.Soc.Clin.Oncol., 13, A713 (1994).

51. W.K. Hong, J. Endicott, L.M. Itri, W. Doos, J.G. Batsakis, R. Bell, S. Fofonoff, R. Byers, F.N. Atkinson, C. Vaughan, B.B. Toth, A. Kramer, I.W.S. Skipper and S. Strong, N.Engl.J.Med., 315, 1501 (1986).
52. F.L. Meyskens, Jr., E. Surwit, T.E. Moon, J.M. Childers, J.R. Davis, R.T. Dorr, C.S. Johnson and D.S. Alberts, J.Natl.Canc.Instit., 86, 539 (1994).
53. W.K Hong, S.M. Lippman, L.M. Itri, D.D. Karp, J.S. Lee, R.M. Byers, S.P. Schantz, A.M. Kramer, R. Lotan, L.J. Peters, I.W. Dimerty, B.W. Brown and H. Goepfert, N.Engl.J.Med., 323, 795 (1990).
54. G. Fountzilas, J.Chemotherapy, 6, 127 (1994).
55. A. Costa, Eur.J.Canc., 29A, 589 (1993).
56. A. Costa, K. Formelli, S. Chisea, A. Decenisi, G. DePalo and U. Veronesi, Canc.Res.(Supp.), 54, 2032 (1994).
57. J. Han, L. Jiao, Y. Lu, Z. Sun, Q.M. Gu and K.J. Scanlon, In Vivo, 4, 153 (1990).
58. M. Igawa, T. Tanabe, G.W. Chodak and D.B. Rukstalis, The Prostate, 24, 299 (1994).
59. M.I. Dawson, P.D. Hobbs, R.B. Stein, T.S. Berger and R.A. Heyman in "Retinoids: Progress in Research and Clinical Applications"; M.A. Livrea and L. Packer, Eds., Marcel Dekker, New York, N.Y., 1993, p. 205.
60. J.M. Lehmann, M.I Dawson, P.D. Hobbs, M. Husmann and M. Pfahl, Cancer Research 51, 4804 (1991).
61. C. Delescluse, M.T. Cavey, B. Martin, B.A. Bernard, U. Reichert, J. Maignan, M. Darmon and B. Shroot, Mol.Pharm., 40, 556 (1991).
62. H. Kagechika, E. Kawachi, Y. Hashimoto, T. Himi and K. Shudo, J.Med.Chem., 31, 2182 (1988).
63. R.A.S. Chandraratna, E. Henry, J. Attard, S.J. Gillett, T. Song, M.E. Garst, S. Nagpal, J. Athanikar, T. Arefieg, D.W.Gil, L.A. Wheeler, D. Lew-Kaya and J. Sefton in "Proceedings of the XIIIth International Symposium on Medicinal Chemistry", Paris, France, 1994, in press.
64. K. Eckhardt and G. Schmitt, Toxicology Letters, 70, 299 (1994).
65. T. Esgleyes-Ribot, R.A.S. Chandraratna, D. Lew-Kaya, J. Sefton and M.J. Duvic, Am. Acad. Dermatol. 30, 581 (1994).
66. B.A. Bernard, J-M. Bernardon, C. Delescluse, B. Martin, M-C. Lenoir, J. Maignan, B. Carpentier, W.R. Pilgrim, U. Reichert and B. Shroot, Biochem.Biophys.Res.Commun., 186, 977 (1992).
67. B. Shroot, B. Martin, M.J. Bernardon, M.T.Cavey, B.A. Bernard, I. Carlavan, B. Charpentier, W.R. Pilgrim and U. Reichert in "Retinoids: Progress in Research and Clinical Applications", M.A. Livrea and L. Packer, Eds., Marcel Dekker, New York, N.Y., 1993, p. 261.
68. J-C. Jamoulle, L. Grandjean, E. Lamaud, B. Shroot and H. Schaefer, J.Invest. Dermat., 94, 731 (1990).
69. J.M. Lehmann, L. Jong, A. Fanjul, J.F. Cameron, X.P. Lu, P. Haefner, M.I. Dawson and M. Pfahl, Science, 258, 1944 (1992).
70. M.F. Boehm, M. McClurg, C. Pathirana, D. Mangelsdorf, S.K. White, J. Hebert, D. Winn, M.E. Goldman and R.A. Heyman, J.Med.Chem., 37, 408 (1994).
71. M.F. Boehm, L. Zhang, B.A. Badea, S.K. White, D.E. Mais, E. Berger, C. Suto, M.E. Goldman and R.A. Heyman, J.Med.Chem., 37, 2930 (1994).
72. A.M. Nadzan, M.F. Boehm, L. Zhang, B.A. Badea, S.K. White, D.E. Mais, E. Berger, C.M. Suto, M.R. McClurg and R.A. Heyman in "Proceedings of the XIIIth International Symposium on Medicinal Chemistry", Paris, France, 1994, in press.
73. V.A. Miller, F.M. Benedetti, J.R. Rigas, W.P. Wong, M.G. Kris, G.M. Gill, J.A. Truglia, E. Ulm, D. Strauss, and R.P. Warrell, Jr., Proc.Annu.Meet.Am.Assoc.Canc.Res., 36, A1445 (1995).
74. L. Jong, J.M. Lehmann, P.D. Hobbs, E. Harlev, J.C. Huffman, M. Pfahl and M.I. Dawson, J.Med.Chem., 36, 2605 (1993).
75. C. Apfel, F. Bauer, M. Crettaz, L. Forni, M. Kamber, F. Kaufmann, P. LeMotte, W. Pirson and M. Klaus, Proc.Natl.Acad.Sci., 89, 7129 (1992).
76. S. Keidel, P. LeMotte and C. Apfel, Mol.Cell.Biol., 14, 287 (1994).
77. S. Kaneko, H. Kagechika, E. Kawachi, Y. Hashimoto and K. Shudo, Med.Chem.Res., 1, 220 (1991).
78. L. Eyrolles, E. Kawachi, Y. Matsushima, O. Nakajima, H. Kagechika, Y. Hashimoto and Koichi Shudo, Med.Chem.Res., 2, 361 (1992).
79. J. Eyrolles, H. Kagechika, E. Kawachi, H. Fukasawa, T. Ijima, Y. Matsushima, Y. Hashimoto and K. Shudo, J.Med.Chem, 37, 1508 (1994).
80. M-O Lee, P.D. Hobbs, X-K Zhang, M.I Dawson and M. Pfahl, Proc.Natl.Acad.Sci., 91, 5632 (1994).
81. R.M. Cesario, M.R. McClurg, G.L. Wong, J.P. Sicurello, R.A. Heyman and W.M. Lamph, Proc.Annu.Meet.Am.Assoc.Canc.Res., 35, A1645 (1994).
82. R. Lotan, M.I. Dawson, C-C Zou, L. Jong, D. Lotan and C-P Zou, Cancer Res., 55, 232 (1995).

Chapter 14. Biological Response Modification by ß-D-Glucans

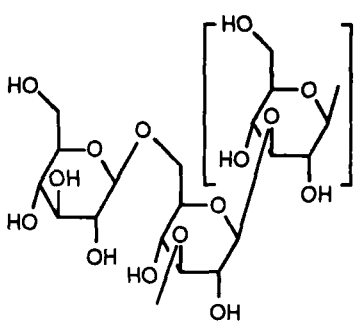
Robert C. Goldman
Abbott Laboratories, Pharmaceutical Research Division D-47M/AP9A
100 Abbott Park Rd., Abbott Park, IL 60064-3500

Introduction - The mammalian immune defense system is a complex and highly evolved assemblage, functioning to defend the organism from constant microbial attack from without, and as well disease from within in the form of emerging tumor cells. Although the immune system in its normal state is quite successful in performing its protective role, the slightest decline in functional capacity can predispose the host to infection, and/or lowering of anti-tumor immune surveillance. Furthermore, specific patient populations with normal immune system function are still at risk for infection in certain clinical settings. Thus exogenous stimulation of immune system function is a logical clinical approach to positive intervention. One approach uses endogenous molecules of the immune system (e.g. various cytokines), while another uses exogenous elements, usually of microbial origin. The current report will focus on fungal glucans and their potential for clinical development.

Background - Zymosan, a cell wall preparation derived from *Saccharomyces cerevisiae*, has diverse physiological effects, many of which can be beneficial. Cell wall glucan was implicated as the active component in zymosan (1), and subsequent studies revealed that ß-glucans in general did evoke many of the beneficial effects, including stimulation of resistance to microbial infection and activation of anti-tumor responses. Although no glucan-derived products are yet approved for clinical use in the United States, glucans are used elsewhere in specific clinical settings. The structure, biological activity, mode of action, preclinical and clinical data pertaining to glucans will be reviewed.

GLUCAN STRUCTURE

General Aspects - Natural product glucans consist of a linear backbone of ß-1,3 linked D-glucopyranosyl groups with varying degrees of branching from the C6 position. Most reported degrees of branching, i.e., the number of branches/main chain residues (DB), should be considered average values with the possibility for heterogeneity in branch distribution as was indicated for glucan isolated from *Volvariella* (2). Branches are usually only a single glucose residue, although more than one glucose unit may be present in some glucans (3,4), and see PGG-glucan and lentinan below. The repeating unit of natural product glucans with varying branching frequencies include: *Alcaligenes* (curdlan), *Saccharomyces* (5), *Omphalia* OR-OL-2c, *Aspergillus* (6), *Agrocybe* (7) and *Euglena* (paramylon), (DB=0), *Omphalia* OL-2-III, **1** (DB=1/24), *Volvariella*, **2** (DB=1/5.5), *Fusarium*, **3** (DB=1/5), *Cryptoporus*, **4** (DB=1/4), *Amanita* glucan, **5**, (DB=2/7), *Grifola* (grifolan), *Omphalia* OL-2-I, *Schizophyllum* (schizophyllan, Schizofiran), and *Sclerotinia* (scleroglucan), **6** (DB=1/3), *Omphalia* OL-2-II, **7** (DB=1/2), and OL-2, **8** (DB=2/3) glucans. Branching frequency can modulate glucan structure and biological activities (see below).



1 n = 23, **2** n = 5.5, **3** n = 4, **4** n = 3, **5** n = 3.5, **6** n = 2, **7** n = 1, **8** n = 0.5

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

The three dimensional structures of lentinan (8), curdlan (9) and 6, schizophyllan, (10) were elucidated by X-ray diffraction studies. Triple helical, parallel strands of glucan are hydrogen bonded via the C2 hydroxyls, with higher order structures arising via hydrogen bonding between C4 and C6 hydroxyls. Since the C6 hydroxyls are on the external surface of the triple helix, branches would project away from the helix surface and likely modulate subtle aspects of glucan structure and biological activity. A stereo view of the glucan triple helix is shown (Figure 1). ß-Glucans can also adopt single chain and single helix forms, as assessed by NMR spectroscopy (11,12). Linear rods, hairpins, circular, globular, and other structures were identified by electron microscopy depending on the glucan sample and mode of preparation (13,14).

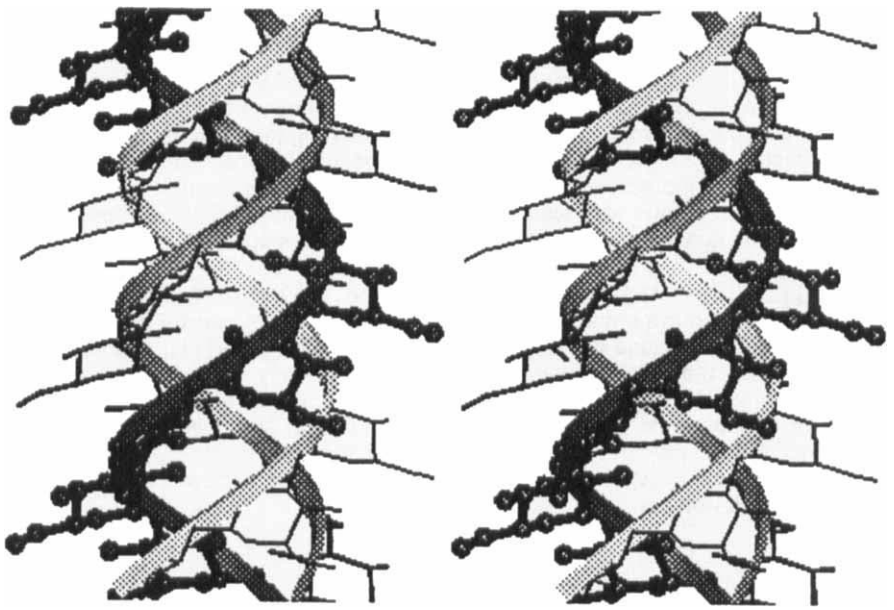


Figure 1. Stereo view of the glucan triple helix.

GLUCAN RECEPTORS

Early in evolution defense systems evolved to detect and rapidly respond to fungal infection. ß-Glucan, a distinguishing component from fungal cells walls, is thus recognized and a defense response initiated. Data are available for specific receptor recognition of glucans in mammalian and non-mammalian systems, resulting in signal transduction events leading to regulated defense responses.

Non Mammalian - The unit glucan structure binding to a plant glucan receptor was determined, and solubilization and identification by photolabeling of the receptor were reported (15-17). A specific heptasaccharide structure is recognized by the receptor (Kd in the nanomolar range), and elicits plant defense responses. Specific glucan binding proteins also exist in fish and invertebrates, and function to trigger defense systems (18-21). A trimer was the minimal structure interacting with the receptor in salmon, and the intact non-reducing terminus was required (22). The crayfish glucan binding protein may have homology to the glucan binding site of ß-glucanase (23).

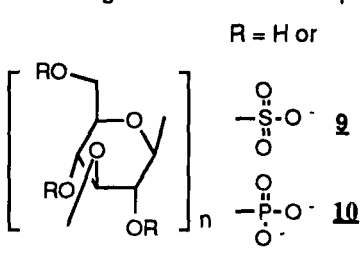
Mammalian - Complement receptor 3 (CR3) and other receptors are implicated in the response of mammalian systems to glucans. CR3 is an integrin receptor known to bind several ligands, and apparently ß-glucans as well (24). A role for CR3 in binding glucan

is supported by the following: 1) antibody against CR3 blocked responses to glucan (25-27), 2) neutrophils from patients with a deficiency of CR3 were unresponsive to β-glucan (26), and 3) CR3 was isolated from neutrophils by affinity chromatography on β-glucan-Sepharose (26). However, CR3 affinity purified on iC3b-Sepharose did not bind zymosan and derived glucan in a solid phase assay (28). In addition, stimulation of CR3 with glucan up regulates CR3 (29), and stimulates NK cell cytotoxic activity (24,27,30) presumably via iC3b coated on target cells. Although CR3 plays a role in glucan induced synthesis of monocyte platelet-activating factor (PAF), the actual receptor interacting with glucan was considered to be distinct from CR3 (31). Glucan interaction with CR3 can alter PKC activity, and in the presence of vanadate, can lead to PLA2 activation (33). In addition, mouse peritoneal macrophages have a receptor which reversibly bound aminated β-1,3 glucan, resulting in glucan internalization (32).

An anti-idiotype (anti-Id) antibody, prepared using a monoclonal antibody against the β-1,3 glucan laminarin coupled to protein (34), was reported to interact with a β-glucan receptor on monocytes. Iodinated anti-Id bound to U937 cells, and reacted with proteins of molecular weights 180 and 160 kDa (35) that consisted of subunits (36) which were differentially regulated (37). A heptasaccharide of undefined structure may represent the unit structure interacting with this receptor (38). NK cell surface proteins which bound yeast wall glucans were also identified in a separate study (39). Corticosteroid treatment of human monocytes increased subsequent phagocytosis of glucan particles as well as glucan stimulated secretion of β-N-acetylglucosaminidase (40), presumably via modulation of glucan receptors. Retinoic acid and 1,25-dihydroxyvitamin D3 also induced β-1,3 glucan receptors on P388D1 cells (41). In addition, crosslinking of receptors may influence cellular responses to glucans (42).

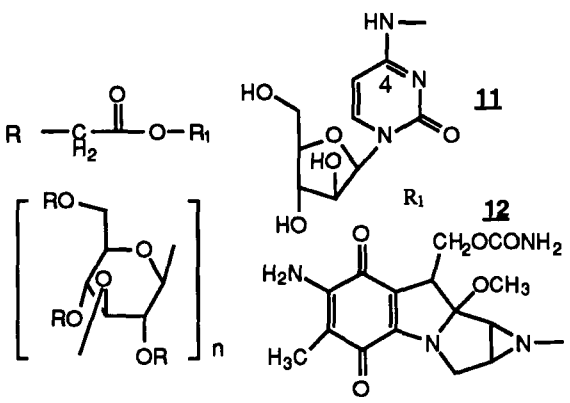
DERIVATIVES OF GLUCANS

Chemically Modified Glucans - Glucan physiochemical properties and biological activity can be modulated by chemical modification. Insoluble yeast glucan was converted to water soluble glucan sulfate, **9**, (43,44) yielding material with repeating unit empirical formulas of $(C_6H_{10}O_5)_8 \cdot 3\ SO_3NH_4$ and $(C_6H_{10}O_5)_5 \cdot 3H_2SO_4$. These modified glucans still formed triple helices in solution, and were capable of stimulating bone marrow proliferation and resistance to microbial challenge in mouse models. Soluble, triple helical glucan phosphate with formula $(C_6H_{10}O_5)_7 \cdot PO_3H_2$, **10**, was also prepared (45,46). Although the extent of substitutions along the β-1,3 backbone was determined, no experimental data were given as to the precise positions of the sulfate or phosphate groups. Both modified glucans contained few if any branches (5). No significant toxicity was observed with glucan phosphate, which is active in promoting wound healing, stimulating resistance to microbial infection, hematopoesis, anti-tumor responses and protection from irradiation (46). β-1,3-Glucans with varying degrees of branching (DB=0, 1/3 and 1/2 see above) were sulfated by reaction with chlorosulfonic acid-pyridine and converted to the sodium salt for analysis of bioactivity (47). Degree of substitution altered the effects of glucans on plasma clotting, complement activation, macrophage stimulation, and mitogenic activity. In addition, the biological effects of a branched glucan were altered by substitution with carboxymethyl groups, suggesting that receptors recognized specific structural features of native glucan (48).



Glucan-Drug Conjugates - Glucan conjugation to cytotoxic drugs modulated toxicity while maintaining the beneficial effects of glucans. Carboxymethylated yeast β-1,3 glucan was coupled to 1-β-D-arabinofuranosylcytosine (araC) (49) by activating the polysaccharide carboxyl group with isobutylchloroformate and formation of a peptide

bond via the 4-NH2 group of araC yielding **11**. This conjugate possessed enhanced anti-leukemic activity, and maintained the immunomodulatory activity of the glucan. Mitomycin C was also conjugated to carboxymethylated, linear ß-1,3 glucan from *Alcaligenes* in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (50) yielding **12**. The conjugate was active against P388 leukemia, and sarcoma 180 solid tumors in mice. Mitomycin C conjugated to carboxymethylated schizophyllan (DB=1/3) was similarly prepared (51). Carboxymethylation was extensive (degree of substitution of 0.87), occurring predominantly at the C4 or C6 hydroxyls. The conjugate was active against sarcoma 180 solid tumors in mice, and caused less reduction of circulating leukocyte numbers compared to mitomycin C alone.



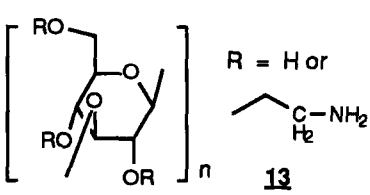
11

12

IN VITRO AND *IN VIVO* RESPONSES TO GLUCANS

ß-glucans affect immune system functions *in vitro* including NK cell cytotoxicity (24,27,30,39), CR3 receptor up-regulation (29), production of cytokines (52-57), PLA2 (33), PGE2 (56), arachidonic acid (58), and wound healing factors (57). The relationship between the induction of these and other factors can influence immune system response to bacterial challenge (59). *In vivo* effects of glucans include:

Stimulation of Resistance to Microbial Challenge - High molecular weight triple helical scleroglucan, **6**, increased resistance of mice to challenge with *Staphylococcus aureus*, *Candida albicans* and mouse hepatitis virus, with little toxicity (60). Glucan phosphate, **10**, was also active in several animal models (46). The N,N-dimethylaminoethyl derivative of paramylon (DB=0) stimulated resistance of mice to challenge with various microorganisms, whereas carboxymethyl paramylon and paramylon sulfate were less effective (61). Yeast derived ß-1,3-glucan also acted synergistically with antibiotics via enhanced IL-1 secretion by macrophages, and enhanced bone marrow proliferation (62). Soluble, aminated ß-1,3 glucan, **13**, modulated cytokine production and protected mice against otherwise lethal bacterial infections (63). Although timing of glucan administration was critical in most studies (also see below for PGG-glucan) long term protection against bacterial challenge was induced in rats (64).



13

Stimulation of Hematopoesis - Glucans also enhanced hematopoesis in animal models, possibly via induction of colony stimulating factors. Scleroglucan, **6**, enhanced bone marrow proliferation when administered to mice (60). Soluble glucan alone, and in combination with human granulocyte colony-stimulating factor enhanced survival and hematopoesis in irradiated mice (65,66). Glucan also abrogated the suppressive effects of pefloxacin on hematopoesis following irradiation (67). Soluble yeast-derived glucan also stimulated hematopoesis with increases in serum levels of granulocyte-macrophage colony stimulating activity (68). Chemically modified glucans (glucan sulfate, **9**, and glucan phosphate **10**) also stimulated murine bone marrow proliferation following intravenous administration (43,46) in mice.

Stimulation of Anti-Tumor Responses - When examined, the anti-tumor activity of glucans appears due to immune system stimulation as opposed to direct activity against tumor cells. Induction of cytokines, and possible activation of cytotoxic cells, are involved (see below). Scleroglucan, **6**, (DB=1/3) increased survival of mice challenged with syngeneic lymphoma, melanoma or adenocarcinoma (60), and inhibited pulmonary metastasis in mice when administered orally or intraperitoneally (69). Glucan also reduced mitomycin C toxicity and promoted an anti-tumor response in a mouse model (51). Branched ß-glucans of MW 10,000 and 20,000, were active against Sarcoma 180 in mice, while a related glucan of MW 6000 was inactive, suggesting molecular weight may influence glucan activity in specific systems (70). Investigation of the mode of action of aminated glucan, **13**, indicated that tumor regression was stimulated via vascular damage to tumor blood flow, and necrosis via functional T cells and TNF (71). A series of branched glucans isolated from Omphalia were tested for anti-tumor activity in the mouse. OL-2, **8**, (DB=2/3) alone or in combination with 5-fluorouracil had activity against solid tumor and ascites forms (72). In addition, derivatives of OL-2 with lesser degrees of branching (**1**, **4**, **7**) showed potent anti-tumor activity against the solid form of sarcoma 180 mice (73). Another branched glucan isolated from *Amanita*, **5**, (DB=2/7) exhibited significant anti-tumor activity against Sarcoma 180 in mice alone and in combination with mitomycin C (3). Carboxymethylation of the linear glucan prepared from *Agrocybe* yielded a product with activity against the solid form of Sarcoma 180 in mice (7).

Stimulation of Cytokines - Cytokine induction by glucans in *in vivo* animal models was reported in several studies. Administration of OL-2, **8**, to mice induced expression of cytokines, including several interleukins, colony stimulating factors, and IL-1 receptor antagonist (IL-1ra) mRNA's (74,75). Schizophyllan, **6**, induced IL-1 alpha mRNA comparable to that by OL-2, **8**, but a weaker level of IL-1 beta mRNA, and did not induce IL-1ra (75). Glucan also acted synergistically with antibiotics via enhanced IL-1 secretion by macrophages, and enhanced bone marrow proliferation (62). Scleroglucan, **6**, (DB=1/3) enhanced IL-1 production (76), soluble aminated glucan modulated cytokine production and enhanced IL-1 and TNF levels (63), and paramylon (DB=0) induced IL-1 and IL-6 (77), when administered to mice.

Stimulation of Wound Healing - Glucans also enhanced wound healing via modulation of the immune system (46,57,78). The strength of healing surgical incisions was increased by I.V. or topical administration of glucan in a rat surgical model, apparently via enhanced macrophage function and increased cross-linking of collagen (57). Glucan also increased the wound healing rate in a mouse second degree burn recovery model, presumably via alteration of macrophage function (78).

Activities of Glucan via Oral Administration - Several studies have demonstrated biological activity of glucans administered orally to animals. Orally administered glucan, extracted from *Candida albicans* (Glucanil, Gluimmun), increased resistance to challenge with *Candida albicans* or *Staphylococcus aureus* in a mouse model (79). Glucan increased polymorphonuclear leukocytes and peripheral monocytes levels, and the *in vitro* candicidal activity of recovered alveolar macrophages and spleen cells. Scleroglucan, **6**, (DB=1/3) increased the number of alveolar macrophages, enhanced their lysosomal enzyme activity, phagocytic activity, and IL-1 production, presumably via effects on Peyer's patch cells (80). The proliferative response of Peyer's patch cells to B and T-cell mitogens was also enhanced (81). Scleroglucan, **6**, (DB=1/3) inhibited pulmonary metastasis in mice when administered orally or intraperitoneally (69).

Affects in Other Biological Systems - Glucan in combination with zinc and pig immunoglobulin reduced the number of migrating ascarid larvae in the lungs of pigs (82). Glucan isolated from *S. cerevisiae* suppressed lesions caused by *Leishmania* in

mice (83), and acted as an adjuvant during immunization with *Leishmania* (84) and *Trypanosoma* (85). Semisoluble ß1,3-carboxymethylglucan increased survival in a mouse model of acute massive hemorrhage (86). Branched glucan decreased the cumulative incidence of diabetes and insulititis in rats, and increased the total numbers of leukocytes in the peripheral blood and spleen (87). Carboxymethylated curdlan (DB=0) or lentinan increased endotoxin clearance in a dose-dependent manner in rabbits (88).

PHARMACOKINETICS AND TOXICITY

Pharmacokinetics of Administered Glucans - The pharmacokinetics of ß-glucans is not well understood, although some data are available. Aminated glucan was derivatized with tyraminyl cellobiose using cyanuric chloride, and radio-iodinated to allow pharmacokinetics studies in mice (89). Smaller molecular weight glucan was found in the kidney and urine, being eliminated more rapidly due to glomerular filtration, while larger glucan was retained primarily within the liver. Tritium labeled scleroglucan, **6**, (DB=1/3) localized to the liver, spleen, kidney and tumor masses in a mouse model (90). When injected into one of a pair of double-grafted tumors, the glucan remained tumor localized but had anti-tumor effects on the distal tumor, presumably via modulation of immune system. Scleroglucan was also found in the blood, liver, and spleen when administered to normal mice, with significant amounts remaining in the liver and spleen for up to four weeks (91). Multiple doses of glucan increased the clearance rate in this system. Radiolabeled *Volvariella*, **2**, glucan (DB=1/6) persisted in the blood (50% of maximum levels still present at 25 hrs), and appeared subject to metabolic conversion in mice to an ionic form which still contained anti-tumor activity (92). The pharmacokinetics of the clinically used anti-cancer adjuvant lentinan (see below) was investigated in normal volunteers and cancer patients (93). Weekly injections of 2 mg lentinan lead to elevated trough levels which might correlate with anti-tumor effects. Blood levels reached a maximum of 50-80 ng/ml at the end of IV infusion (1 mg over 2 hrs), and decreased gradually thereafter (94). Grifolan, **6**, (DB=1/3) administered to mice also accumulated at tumor sites, and in various organs, including the spleen (95). Two phases of elimination ($T_{1/2}$ = 0.5 to 1 hr, and 7 to 12 hrs) were observed following I.V. administration of PGG-glucan to normal volunteers (96), and see below.

Toxicity of Glucans - In general, soluble glucan preparations appear less toxic than particulate glucans. Soluble glucan phosphate derived from *S. cerevisiae* showed little acute or chronic animal toxicity in preclinical animal evaluations (46,97). A yeast derived glucan was associated with transient hepatomegaly and associated histologic changes, when tested in mice at 250 mg/kg I.V. (98). In contrast, systemic administration of microparticulate glucan can cause granuloma formation, hepatosplenomegaly, microembolism, and sensitization to endotoxin (46). Toxicity of a single injection of microparticulate glucan correlated with chronic murine hepatitis infection (99), with minimal effects on virus free mice, indicating that the immune system state my affect responses to glucans. Many of the other *in vivo* studies referenced above also reported little toxicity for soluble glucan preparations.

HUMAN PRECLINICAL AND CLINICAL DATA

Although available for only small numbers of patients, data on the clinical efficacy of specific ß-1,3 glucans, when used in trauma and surgery patients who are at risk for microbial infection, and as a combination therapy for the treatment of cancer, are available.

PGG-Glucan - Clinical development of a genetically modified *Saccharomyces cerevisiae* glucan, PGG-glucan (Betafectin), is in progress (see below). The precise structure of the material prepared from cells is not known, but certain structural

features were described. The altered ß-1,3 glucan, **14**, had DB=1/3.4 (100) to DB=1/2 (101), and the average length of the side chains, which consisted of more than one glucosyl unit connected by ß-1,6 linkages, were 1.1 (100) to 3 (101). PGG-Glucan was proposed to act via interaction with ß-glucan receptors on monocytes and neutrophils, stimulating production of cytokines, inflammatory, and colony stimulating factors (101). This conclusion correlated with the effects of other ß-1,3 glucans (see above). Most recently (96), the PGG-glucan used in clinical trails was described as a triple helical, branched ß-1,3 glucan (no branching frequency given), possessing branches with ß-1,3 linkages, **15**. Immune system priming, as opposed to direct activation of immune system function and cytokine induction is now considered to be the mechanism of action of PGG-glucan (96,102). Administration to mice stimulated increased resistance to bacterial challenge in early studies (103). PGG-Glucan also enhanced microbicidal activity of human peripheral-blood monocytes and neutrophils (104), nitric oxide production from rat neutrophils (105), and stimulated hematopoesis alone (106) and in combination with GM-CSF (107). PGG-Glucan mode of action presumably involves binding to a ß-glucan receptor (see above section).

14 where R =

n = 1 - 3.4; n_1 = 1 - 3

R

15 where R =

n and n_2 not given

Stimulation of Resistance to Bacterial Infection in Patients at Risk - Two recent double blind, placebo-controlled, randomized phase 2 studies examined the safety and efficacy of PGG-glucan in preventing postoperative infection in patients undergoing major thoracic or abdominal surgery. In the first trial results for 17 patients receiving multiple doses (0.5 mg/kg) of PGG-glucan were compared to placebo administration (96). Infection incidence and severity was lower in PGG-glucan treated patient group, as was the incidence of postoperative antibiotic usage, and duration of hospital stay including days in intensive care. In a second study, 51 patients in the treatment group were divided into three groups receiving 0.1, 0.5 1.0 or 2.0 mg/kg doses (102). Trends toward reduced infection incidence were found with increasing dosage, and data were further analyzed by pooling data for specific groups (see original article for methods of analysis). Two other independent studies reported efficacy of yeast glucan administration to trauma patients. Yeast derived glucan (30 mg I.V. every 12 to 24 hours) decreased the incidence of pneumonia and sepsis in a group of 21 patients treated in a randomized, double blinded, controlled trial (108). The incidence of antibiotic usage was also decreased, as was mortality due to infection and hospitalization stay, including days in intensive care. Another prospective, randomized, double blinded study examined the effects of yeast derived glucan phosphate, **10**, administration to 21 trauma patients given 50 mg /square meter IV daily for seven days (109). Septic morbidity was reduced in the treatment group, as was the mortality rate, and slight increases in IL2, but not TNF levels were transiently observed.

Stimulation of Anti-Tumor Responses - Lentinan is a purified polysaccharide of 500 kDa extracted from *Lentinus edodes*, composed of a linear ß-1,3 glucan with ß-1,6-linked branches (DB=1/12.5), with evidence of branches of greater that one glucosyl unit, and some internal ß-1,6 linkages within the main chain (110). Positive responses were observed when lentinan was used in conjunction with a chemotherapeutic agent for breast (111), colo-rectal (111), gastric (110,111), prostate (112) cancers, and malignant ascites and pleural effusions produced from end-stage anal, esophageal, gastric, and pancreatic cancers (113). No positive effects were observed when lentinan was combined with 5FU in the treatment of hepatocellular carcinoma (114). Administration of lentinan stimulates macrophages to produce increased amounts of colony stimulating factors, IL-I, IL-III, and other serum factors which modulate activity of the immune system. Sizofiran, **6**, (DB=1/3) is a purified polysaccharide of 450 kDa isolated from *Schizophyllum*. Sizofiran was used in conjunction with radiotherapy for stage II and stage III cervical cancer (115-117). The response rate was greatest for stage II cancer (116,117), with no significant difference observed for stage III cancer.

FUTURE DIRECTIONS

Our knowledge of the biology and chemistry of ß-glucans has progressed steadily towards an understanding of 1) glucan structure vs activity, 2) mechanisms of glucan interaction with the immune system and *in vivo* efficacy, and 3) potential for clinical use. However, even though basic structural properties of natural product glucans are known, they are not structurally defined in the context of the precise structural features interacting with the immune system receptor(s), and the receptor(s) to which glucans bind have not be rigorously defined. The identification of CR3 and other receptor polypeptides for glucans in mammalian systems should ultimately allow a detailed study of glucan structure vs its ability to interact with the immune system, and formulation of an explicit frame work to guide future studies.

REFERENCES

1. S.J. Riggi and N.R. Di Luzzio, Am.J.Physiol., 200, 297 (1961).
2. E. Kishida, Y. Sone and A. Misaki, Carbohydr.Res., 193, 227 (1989).
3. T. Kiho, M. Katsuragawa, K. Nagai, S. Ukai and M. Haga, Carbohydr.Res., 224, 237 (1992).
4. J. Perret, M. Bruneteau and G. Michel, Carbohydr.Polym., 17, 231 (1992).
5. H.E. Ensley, B. Tobias, H.A. Pretus, R.B. McNamee, E.L. Jones, I.W. Browder and D.L. Williams, Carbohydr.Res., 258, 307 (1994).
6. P. Rouhier, M. Bruneteau, G. Michel, J. Fiema and A. Zurzycka, Carbohydr.Res., 262, 155 (1994).
7. T. Kiho, I. Yoshida, K. Nagai, S. Ukai and C. Hara, Carbohydr.Res., 189, 273 (1989).
8. T.L. Bluhm and A. Sarko, Can.J.Chem., 55, 293 (1977).
9. Y. Deslandes, R.H. Marchessault and A. Sarko, Macromol., 13, 1466 (1980).
10. Y. Takahashi, T. Kobatake and H. Suzuki, Rep.Prog.Polym.Phys.Jap., 27, 767 (1984).
11. Y. Yoshioka, N. Uehara and H. Saito, Chem.Pharm.Bull., 40, 1221 (1992).
12. H. Saito, Y. Yoshioka, M. Yokoi and J. Yamada, Biopolym., 29, 1689 (1990).
13. B.T. Stokke, A. Elgsaeter, C. Hara, S. Kitamura and K. Takeo, Biopolym., 33, 561 (1993).
14. S. Kitamura, T. Hori, K. Kurita, T. Kenichi, C. Hara, W. Itoh, K. Tabata, A. Elgsaeter and B.T. Stokke, Carbohydr.Res., 263, 111 (1994).
15. D. Mohnen and M.G. Hahn, Semin.Cell Biol., 4, 93 (1993).
16. A. Darvill, C. Augur, C. Bergmann, R.W. Carlson, J.J. Cheong, S. Eberhard, M.G. Hahn, V.M. Lo, V. Marfa, B. Meyer, D. Mohnen, M.A. O'Neal, M.D. Spiro, H. van Helbeeck, W.S. York and P. Albersheim, Glycobiol., 2, 181 (1992).
17. E.G. Cosio, T. Frey and J. Ebel, Eur.J Biochem, 204, 1115 (1992).
18. A.J. Ainsworth, Vet.Immunol.Immunopathol., 41, 141 (1994).
19. M.W. Johansson and K. Soderhall, Dev.Comp.Immunol., 17, 495 (1993).
20. B. Duvic and K. Soderhall, Eur.J.Biochem, 207, 223 (1992).
21. P.O. Thornqvist, M.W. Johansson and K. Soderhall, Dev.Comp.Immunol., 18, 3 (1994).
22. R.E. Engstad and B. Robertsen, Develop.Comp.Immunol., 18, 397 (1994).
23. L. Cerenius, Z. Liang, B. Duvic, P. Keyser, U. Hellman, E. Palva, S. Iwanaga and K. Soderhall, J.Biol.Chem., 269, 29462 (1994).
24. G.D. Ross and V. Vetvicka, Clin.Exp.Immunol., 92, 181 (1993).

25. J.A. Cain, S.L. Newman and G.D. Ross, Complement, 4, 75 (1987).
26. G.D. Ross, J.A. Cain, B.L. Myones, S.L. Newman and P.J. Lachmann, Complement, 4, 61 (1987).
27. L. Di Renzo, E. Yefenof and E. Klein, Eur.J.Immunol., 21, 1755 (1991).
28. J.A. Van Strijp, D.G. Russell, E. Tuomanen, E.J. Brown and S.D. Wright, J.Immunol., 151, 3324 (1993).
29. S. Muto, V. Vetvicka and G.D. Ross, J.Clin.Immunol., 13, 175 (1993).
30. E. Klein, L. Di Renzo and E. Yefenof, Mol.Immunol., 27, 1343 (1990).
31. M.R. Elstad, C.J. Parker, F.S. Cowley, L.A. Wilcox, T.M. McIntyre, S.M. Prescott and G.A. Zimmerman, J.Immunol., 152, 220 (1994).
32. Z. Konopski, B. Smedsrod, R. Seljelid and T. Eskeland, Biochim.Biophys.Acta, 1221, 61 (1994).
33. R. Goldman, E. Ferber, R. Meller and U. Zor, Biochim.Biophys.Acta, 1222, 265 (1994).
34. J.K. Czop, M.F. Gurish and J.L. Kadish, J.Immunol., 145, 995 (1990).
35. J.K. Czop and J. Kay, J.Exp.Med., 173, 1511 (1991).
36. J.K. Czop and T. Szabo, FASEB J., 8, A388 (1994).
37. T. Szabo, J.L. Kadish and J.K. Czop, FASEB J., 8, A214 (1994).
38. M.J. Janusz, K.F. Austen and J.K. Czop, J.Immunol., 142, 959 (1989).
39. X. Duan, M. Ackerly, E. Vivier and P. Anderson, Cell Immunol., 157, 393 (1994).
40. J. Kay and J.K. Czop, Immunol., 81, 96 (1994).
41. R. Goldman, Immunol., 63, 319 (1988).
42. D.D. Poutsiaka, M. Mengozzi, E. Vannier, B. Sinha and C.A. Dinarello, Blood, 82, 3695 (1993).
43. D.L. Williams, H.A. Pretus, R.B. McNamee, E.L. Jones, H.E. Ensley and I.W. Browder, Carbohydr.Res., 235, 247 (1992).
44. D.L. Williams, H.A. Pretus, R.B. McNamee, E.L. Jones, H.E. Ensley, I.W. Browder and N.R. DiLuzio, Immunopharmacol., 22, 139 (1991).
45. D.L. Williams, R.B. McNamee, E.L. Jones, H.A. Pretus, H.E. Ensley, I.W. Browder and N.R. DiLuzio, Carbohydr.Res., 219, 203 (1991).
46. D.L. William and I.W. Browder, Polymers Adv.Tech., 5, 529 (1994).
47. T. Suzuki, N. Ohno, Y. Adachi, A.F. Cirelli, J.A. Covian and T. Yadomae, J.Pharmacobiodyn., 14, 256 (1991).
48. Y. Adachi, N. Ohno and T. Yadomae, Biol.Pharm.Bull., 16, 462 (1993).
49. V. Kery, L. Novotny, K. Tihlarik, J. Haplova, M. Kacurakova, J. Sandula and E. Balazova, Int.J.Biochem., 22, 1203 (1990).
50. K. Nagai, J. Tanaka, T. Kiho and S. Ukai, Chem.Pharm.Bull.(Tokyo)., 40, 986 (1992).
51. S. Usui, K. Murashima, M. Sakai, T. Kiho and S. Ukai, Biol.Pharm.Bull.(Tokyo)., 17, 1165 (1994).
52. G. Abel and J.K. Czop, Int.J.Immunopharmacol., 14, 1363 (1992).
53. Z. Konopski, R. Seljelid and T. Eskeland, Scand.J.Immunol., 40, 57 (1994).
54. Z. Konopski, R. Seljelid and T. Eskeland, Scand.J.Immunol., 37, 587 (1993).
55. L.T. Rasmussen Busund, Z. Konopski, P. Oian and R. Seljelid, Microbiol.Immunol., 36, 1173 (1992).
56. M. Doita, L.T. Rasmussen, R. Seljelid and P.E. Lipsky, J.Leukoc.Biol., 49, 342 (1991).
57. W. Browder, D. Williams, P. Lucore, H. Pretus, E. Jones and R. McNamee, Surgery, 104, 224 (1988).
58. T. Daum and M.S. Rohrbach, FEBS Lett., 309, 119 (1992).
59. L.T. Rasmussen, J. Fandrem and R. Seljelid, Scand.J.Immunol., 32, 333 (1990).
60. H.A. Pretus, H.E. Ensley, R.B. McNamee, E.L. Jones, I.W. Browder and D.L. Williams, J Pharmacol.Exp.Ther., 257, 500 (1991).
61. H. Sakagami, K. Kikuchi, M. Takeda, T. Sato, S. Ichikawa, M. Fujimaki, C. Wada and N. Komatsu, In Vivo, 5, 101 (1991).
62. W. Browder, D. Williams, E. Sherwood, R. McNamee, E. Jones and N. DiLuzio, Surgery, 102, 206 (1987).
63. L.T. Rasmussen and R. Seljelid, J.Cell Biochem., 46, 60 (1991).
64. S.M. Almdahl and R. Seljelid, Res.Exp.Med.(Berl)., 187, 369 (1987).
65. M.L. Patchen, T.J. MacVittie, B.D. Solberg and L.M. Souza, Exp.Hematol., 18, 1042 (1990).
66. J.R. Maisin, C. Albert and A. Henry, Radiat.Res., 135, 332 (1993).
67. M.L. Patchen, I. Brook, T.B. Elliott and W.E. Jackson, Antimicrob.Agents Chemother, 37, 1882 (1993).
68. M.L. Patchen and T.J. MacVittie, J.Immunopharmacol., 8, 407 (1986).
69. I. Suzuki, T. Sakurai, K. Hashimoto, S. Oikawa, A. Masuda, M. Ohsawa and T. Yadomae, Chem.Pharm.Bull.(Tokyo)., 39, 1606 (1991).
70. W. Blaschek, J. Kasbauer, J. Kraus and G. Franz, Carbohydr.Res., 231, 293 (1992).
71. R. Seljelid, Scand.J.Immunol., 29, 181 (1989).
72. N. Ohno, T. Miura, K. Saito, M. Nishijima, T. Miyazaki and T. Yadomae, Chem.Pharm.Bull.(Tokyo)., 40, 2215 (1992).
73. K. Saito, M. Nishijima, N. Ohno, T. Yadomae and T. Miyazaki, Chem.Pharm.Bull.(Tokyo)., 40, 261 (1992).

74. N. Ohno, K. Saito, J. Nemoto, S. Kaneko, Y. Adachi, M. Nishijima, T. Miyazaki and T. Yadomae, Biol.Pharm.Bull., 16, 414 (1993).
75. J. Nemoto, N. Ohno, K. Saito, Y. Adachi and T. Yadomae, Biol.Pharm.Bull.(Tokyo)., 17, 948 (1994).
76. T. Sakurai, N. Ohno and T. Yadomae, Chem.Pharm.Bull.(Tokyo)., 40, 2120 (1992).
77. Y. Kondo, A. Kato, H. Hojo, S. Nozoe, M. Takeuchi and K. Ochi, J.Pharmacobiodyn., 15, 617 (1992).
78. Y.L. Chen, Chung.Hua.Cheng.Hsing.Shao.Shang.Wai.Ko.Tsa.Chih., 8, 54 (1992).
79. A. Nicoletti, G. Nicoletti, G. Ferraro, G. Palmieri, P. Mattaboni and R. Germogli, Arzneimittelforschung, 42, 1246 (1992).
80. T. Sakurai, K. Hashimoto, I. Suzuki, N. Ohno, S. Oikawa, A. Masuda and T. Yadomae, Int.J Immunopharmacol., 14, 821 (1992).
81. K. Hashimoto, I. Suzuki and T. Yadomae, Int.J.Immunopharmacol., 13, 437 (1991).
82. M. Benkova, Z. Boroskova, J. Soltys, J. Dubaj and S. Szechenyi, Vet.Parasitol., 41, 157 (1992).
83. R. Goldman and C.L. Jaffe, Parasite Immunol., 13, 137 (1991).
84. R.M. Lasarow, D.L. Williams and J.H. Theis, Int.J.Immunopharmacol., 14, 767 (1992).
85. W. Browder and D. Williams, J.Natl.Med.Assoc., 80, 531 (1988).
86. E.I. Vereschagin, T.A. Korolenko and S.A. Arkhipov, Agents Actions Suppl, 38, 385 (1992).
87. K. Kida, T. Inoue, Y. Kaino, Y. Goto, M. Ikeuchi, T. Ito, H. Matsuda and R. Elliott, Diabetes Res.Clin.Pract., 17, 75 (1992).
88. M. Yokota, T. Tsujinaka, J. Kambayashi, M. Watase, M. Sakon and T. Mori, Int.Surg., 76, 255 (1991).
89. B. Smedsrod and R. Seljelid, Immunopharmacol., 21, 149 (1991).
90. M. Suda, N. Ohno, Y. Adachi and T. Yadomae, Biol.Pharm.Bull.(Tokyo)., 17, 131 (1994).
91. M. Suda, N. Ohno, Y. Adachi and T. Yadomae, J.Pharmacobiodyn., 15, 417 (1992).
92. Y. Sone, S. Shibata, E. Kishida and A. Misaki, J.Nutr.Sci.Vitaminol., 40, 335 (1994).
93. Y. Yajima, J. Satoh, T. Kikuchi, I. Fukuda, T. Toyota, H. Saito, N. Okabe and H. Suzuki, Gan.To.Kagaku.Ryoho., 16, 2633 (1989).
94. Y. Yajima, J. Satoh, I. Fukuda, T. Kikuchi and T. Toyota, Tohoku.J.Exp.Med., 157, 145 (1989).
95. T. Takeyama, I. Suzuki, N. Ohno, S. Oikawa, K. Sato, M. Ohsawa and T. Yadomae, J.Pharmacobiodyn., 11, 381 (1988).
96. T.J. Babineau, P. Marcello, W. Swailis, A. Kenler, B. Bistrian and R. Forse, Ann.Surgery, 220, 601 (1994).
97. D.L. Williams, E.R. Sherwood, I.W. Browder, R.B. McNamee, E.L. Jones and N.R. Di Luzio, I nt.J.Immunopharmacol., 10, 405 (1988).
98. W.H. Baker, J.B. Nold, M.L. Patchen and W.E. Jackson, Proc.Soc.Exp.Biol.Med., 201, 180 (1992).
99. J. Vacha, V. Znojil, M. Pospisil, J. Hola and I. Pipalova, Int.J.Immunopharmacol., 16, 51 (1994).
100. S. Jamas, Y.J. Chen, C.H. von der Osten, A.J. Sinskey and C.K. Rha, Carbohyd.Polym., 13, 207 (1990).
101. S. Jamas, D.D. Easson, G.R. Ostroff and A.B. Onderdonk, ACS Symp.Series, 469, 44 (1991).
102. T.J. Babineau, A. Hackford, A. Kenler, B. Bistrian, R.A. Forse, P.G. Fairchild, S. Heard, M. Keroack, P. Caushaj and P. Benotti, Arch.Surg., 129, 1204 (1994).
103. A.B. Onderdonk, R.L. Cisneros, P. Hinkson and G. Ostroff, Infect.Immun., 60, 1642 (1992).
104. W. Macklin, D. Brunke-Reese, Y. Gu, C. Crotty and L. Flsette, FASEB J., 8, A488 (1994).
105. D. Brunke-Reese and W.N. Macklin, FASEB J., 8, A216 (1994).
106. G. Ostroff, R. Nicolosi, C. Yoganathan, C. Rosenfled, J. Neumanitis and H. Leahy, FASEB J., 8, A640 (1994).
107. E. Wakshull, H. Correira and T. Sidloski, FASEB J., 8, A640 (1994).
108. J. de Felippe Junior, S.J. da Rocha, F.M. Maciel, A. Soares and N.F. Mendes, Surg.Gynecol.Obstet., 177, 383 (1993).
109. W. Browder, D. Williams, H. Pretus, G. Olivero, F. Enrichens, P. Mao and A. Franchello, Ann.Surg., 211, 605 (1990).
110. T. Taguchi, Cancer Detect.Prevent.Supp., 1, 333 (1987).
111. G. Chihara, Devop.Biol.Standards, 77, 191 (1992).
112. K. Tari, I. Satake, K. Nakagomi, K. Ozawa, F. Oowada, Y. Higashi, T. Negishi, T. Yamada, H. Saito and K. Yoshida, Hinyokika-Kiyo, 40, 119 (1994).
113. O. Masaaki, Y. Shigefumi, H. Shoichi, S. Kouji and S. Takashi, Biother., 5, 107 (1992).
114. S. Toshiyuki, F. Shinsaku, M. Noriko, W. Yasuhiro, S. Daisuke, Y. Yutaka and S. Yu, Cancer Chemother.Pharmacol., 33, S145 (1994).
115. K. Okamura, Y. Hamazaki, A. Yajima and K. Noda, Biomed.Pharmacother., 43, 177 (1989).
116. N. Kiichiro, T. Shoshichi, Y. Akira, A. Kiyoshi, K. Tatsuhiro, T. Yutaka, O. Mitsuru, S. Kaoru, S. Hajime, H. Shozo and O. Nobuya, Jap.J.Clin.Oncol., 22, 17 (1992).
117. K. Okamura, M. Suzuki, T. Chihara, A. Fijiwara, T. Fukuda, S. Goto, K. Ichinohe, S. Jimi, T. Kasamatsu, N. Kawai, K. Mizuguchi, S. Mori, H. Nakana, K. Noda, K. Sekiba, K. Suzuki, T. Suzuki, K. Takahashi, K. Takeuchi, S. Takeuchi, A. Yajima and N. Ogawa, Cancer, 58, 865 (1986).

Chapter 15. Antiviral Agents

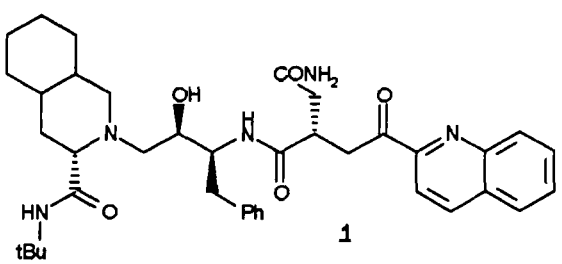
Richard E. Boehme, Alan D. Borthwick, and Paul G. Wyatt
Glaxo Research & Development Ltd.
Stevenage, Hertfordshire SG1 2NY, UK

Introduction - This chapter focuses upon developments in therapies for viral disease, and is divided into three sections: rational design of viral protease inhibitors, immunotherapy of viral diseases, and new antiviral agents.

VIRAL PROTEASE INHIBITORS

HIV Protease - The aspartyl protease (HIV PR) of the human immunodeficiency virus (HIV) is a dimeric enzyme composed of two identical polypeptide chains that associate with twofold symmetry (1). HIV PR has emerged as an important target for chemotherapeutic intervention because of its key role in cleaving the HIV gag-pol polyprotein during viral assembly and budding. Inhibition of HIV PR prevents the maturation of HIV in cell culture (2,3). Time of addition studies suggest that HIV PR inhibitors also act at an early stage of infection, possibly inhibiting cleavage of the nucleocapsid protein and regulation of NEF (4,5). To date, numerous inhibitors of HIV PR with diverse structural features have been reported, but few have been studied extensively in humans, primarily due to poor oral bioavailability.

The peptidomimetic compound Ro31-8959 (**1**) was the first HIV PR inhibitor to exhibit clinical efficacy (6,7). Interestingly, treatment *in vitro* of HIV-infected cells with Ro31-8959 for three months cleared the virus from the cells, suggesting a possible clinical advantage of long-term dosage with HIV PR inhibitors (8). Several HIV PR inhibitors have now entered clinical trials. However, virus resistant to HIV PR inhibitors can be selected rapidly (9-11), so HIV PR inhibitors are now being investigated in combination with HIV inhibitors with other molecular targets (12,13).

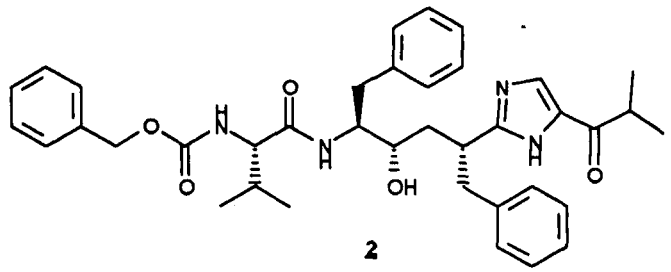


1

The search for HIV PR inhibitors has highlighted the power of coupling X-ray crystallography and computational chemistry. Crystal structures of HIV PR bound to inhibitors and the ability to model these interactions provide a more rational understanding of structure-activity relationships. Rational design processes have been used to introduce groups that impart potency and improve pharmacokinetic profiles. Several approaches have been used: modification of peptide leads based on the natural protease cleavage sites, modification of non-peptidic compounds identified by high throughput screening against HIV PR, and *de novo* design of inhibitors using HIV PR crystallographic data. The wealth of information obtained from such studies will clarify the fundamental nature of molecular interactions and assist rational drug design in the future (14-16).

Copyright © 1998 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Many peptide-based HIV PR inhibitors are transition-state analogues, some exhibiting low nanomolar or subnanomolar enzyme inhibitory potency (17-19). Pseudosymmetric and C_2 symmetric inhibitors which take advantage of the C_2 symmetric nature of the enzyme have also been reported. Although the scissile amide bond is replaced with an isostere in these compounds, most still contain a number of labile bonds due to their peptidic nature. They often retain substantial peptide character, and thus show poor aqueous solubility, oral bioavailability and pharmacokinetic properties. Instability associated with this class of compounds is being addressed by approaches such as replacing the natural amino acid residues with unnatural D-amino acids, and replacement of the amide bonds with small heterocyclic groups (20-22).



2

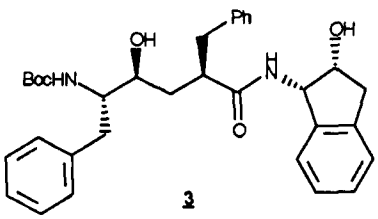
The P_1'-P_2' amide bond was replaced in a hydroxyethylene-based HIV PR inhibitor by thiazole, triazole and imidazole moieties (22). All replacements gave active compounds, but the imidazoles were most potent due to their high basicity promoting hydrogen bond formation. The imidazole derivatives were optimised to afford **2** (Ki_{app} = 0.6nM, HIV IC_{50} = 100nM).

Most work to design HIV PR inhibitors has involved replacement of amino acid residues with surrogates. L-685,434 (**3**), an example of hydroxyethylene dipeptide isostere HIV PR inhibitors, is highly potent, but lacks aqueous solubility and acceptable pharmacokinetics. The decahydroisoquinoline *tert*-butylamide of **1** was combined with the right-hand side of L-685,434 to add an amine group for aqueous solubility and to supply a conformationally restricted P_1/P_2 ligand (23). The resulting compounds **3** and **4** were modelled into the crystal structure of HIV PR. The minimised structures were similar, with the right-hand sides almost superimposed in the P_1' and P_2' groups. The P_1 (Phe) moiety and the P_2 (Boc) group of **4** occupy the same 3-dimensional space as the P_1 decahydroisoquinoline and *tert*-butylamide in **3**. **4** may also bind to water in the flap of HIV PR crystals. This compound did indeed inhibit HIV PR (IC_{50} = 7.6nM), however it was relatively weak in a cell-based assay (IC_{95} = 400nM).

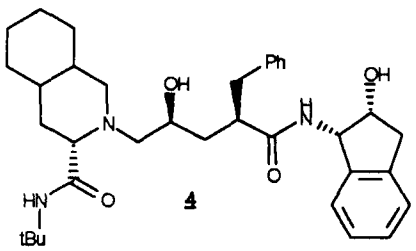
Because **4** showed improved pharmacokinetics compared to **3**, other amine-containing analogues were made. The decahydroisoquinoline ring system was replaced by the piperazine ring (**5**) to afford two possible advantages. The piperazine ring nitrogen could increase solubility and thereby improve pharmacokinetics. The nitrogen is also easily functionalised to introduce a P_3 ligand which could balance the hydrophobic and hydrophilic attributes required for potent activity combined with good pharmacokinetics.

Adding an N4 benzyloxycarbonyl group on the piperazine ring produced a potent inhibitor of HIV PR and of HIV replication *in vitro* (**5**). Crystallography data were consistent with modelling results. P_2 and P_2' ligands bound tightly into the S_2 and S_2' region of HIV PR, where the critical water molecule bridged the two carbonyls of the P_2 and P_1' ligands and the benzyloxycarbonyl moiety filled the lipophilic S_3 domain. Compounds resulting from replacing the benzyloxycarbonyl group with sulphonyl and acyl substituents were potent HIV PR inhibitors, but activity in cell-based assays was

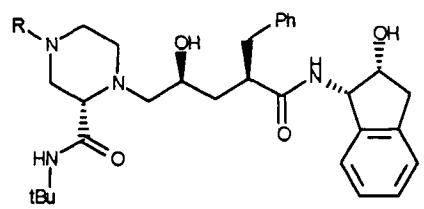
inconsistent due to variable cell penetration. Alkylated piperazines with various arylmethyl substituents were more potent *in vitro*. The ability to modify P_3 without adversely affecting potency allowed optimisation of physical properties. The 3-pyridylmethyl group provided lipophilicity to enhance protease binding and a weakly basic amine to improve aqueous solubility. L-735.524 (**6**) inhibited HIV-1 PR and HIV-2 PR competitively (K_i = 0.52nM and 3.3nM respectively) and in a cell-based antiviral assay yielded an IC_{95} of 50nM. Treatment of HIV-infected patients reduced viraemia by ~95% for 4 weeks, but after 6 months viraemia returned to control levels. Isolated virus was about 4-fold less susceptable than wild-type virus to **6** (24).



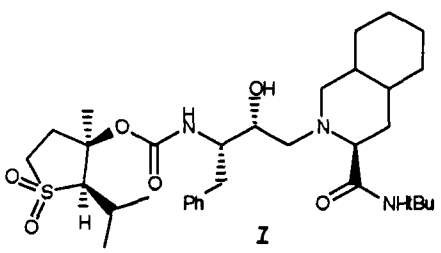
3



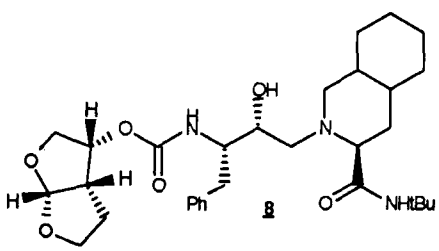
4



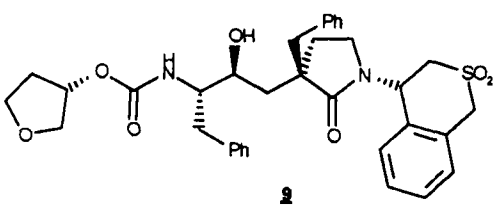
R= tBoc **5**
3-pyridylmethyl **6**



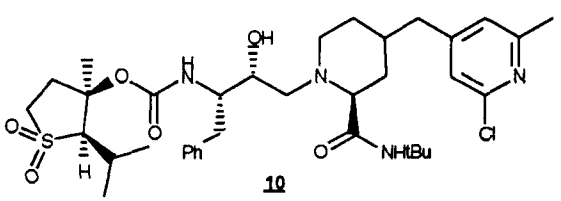
7



8



9



10

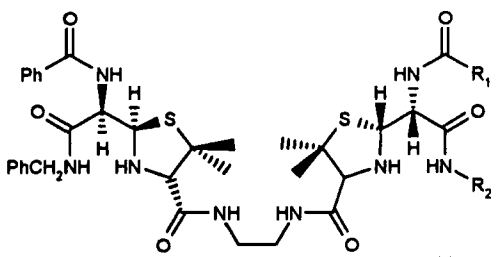
Replacing the P_2 asparagine and the P_3 quinoline with a urethane of (3S)-tetrahydrofuran as a P_2 ligand afforded a compound of moderate activity (25). Optimization of this lead identified the hydroxysulfolane derivative (**7**) as the most potent (HIV PR IC_{50} = 3.5nM). Modelling suggested that the activity of **7** was due to its isopropyl group being optimal to fill the shallow hydrophobic cavity in the S_2 region of the HIV PR binding site. Also, the sulfolane oxygen *cis* to the isopropyl group was within hydrogen bonding distance (2.1Å) to Gly48. In a similar study the quinaldic acid asparagine amide fragment of **1** was replaced with a fused bis-tetrahydrofuran moiety

(26). The optimised compound **8** (HIV PR IC_{50} = 1.8nM; HIV IC_{95} = 46nM) was equipotent to **1** in comparative *in vitro* antiviral tests. Moreover, the substitutions improved aqueous solubility, lowered logP value and reduced molecular weight.

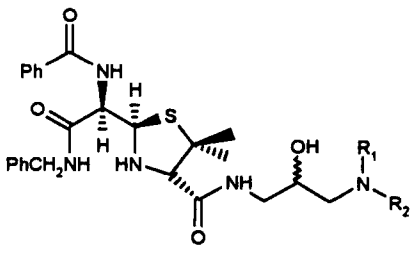
A molecular model of **3** docked into HIV PR indicated that the conformation of the compound was ideal to incorporate the amide bond into a 5-membered lactam ring (27). This modification gave a compound with reduced activity compared with the acyclic analogue, however optimisation of the series led to **8** (IC_{50} = 1.8 nM).

The C_2 symmetry of HIV PR led to combination of the new moieties **5-7** to derive a novel series of HIV PR inhibitors (28). The C_2 symmetry of HIV PR allowed the piperazine carboxamide moiety at the N-terminus of **6** to be transferred to the C-terminus and replace the decahydroisoquinoline *tert*-butylamide group of **7**. The versatile piperazine carboxamide moiety was used again to fine-tune the attributes of the new hybrid molecule to afford the 3,5-disubstituted pyridine derivative (**10**).

To circumvent the problems encountered with many peptidic HIV PR inhibitors, the potential of non-peptidic compounds has been investigated. Several different approaches have been taken, such as high throughput HIV PR screening to identify leads, computation-aided modification of known inhibitors and *de novo* design using the important residues and interactions of the HIV PR active site.



11

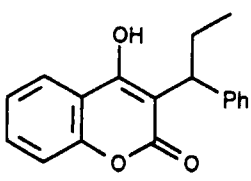


12

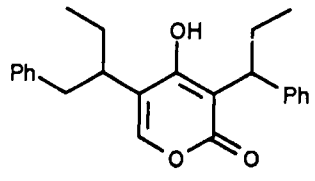
High throughput screening identified the penicillin-derived C_2 symmetric dimers (**11**) as potent HIV PR inhibitors (29). The crystal structure of **11** bound to HIV PR allowed the computer-aided design of the potent inhibitor **12** (HIV PR IC_{50} = 4.6nM). However the high molecular weight and lipophilicity of these series caused poor pharmacokinetic profiles (30). Attempts to improve pharmacokinetics of the symmetrical inhibitors by modification of the P_3/P_3' positions gave compounds with nanomolar HIV PR inhibitory activity and a range of logP values, but none of the compounds exhibited improved pharmacokinetics. Replacement of the statine dipeptide isostere of **12** with a variety of groups including nitrogen-containing ring systems afforded compounds with good HIV PR inhibitory activity. However despite these changes, none of the compounds displayed acceptable pharmacokinetic properties *in vivo*.

High throughput screening by two groups identified compounds containing the 4-hydroxybenzopyran-2-one and 4-hydroxypyran-2-one residues (31,32). Both groups found that the 4-hydroxypyran-2-one residue could span the HIV PR active site. A crystal structure of **13** bound to HIV PR showed that the 4-hydroxy group bound to Asp25 and Asp125, displacing the catalytic water molecule (31). The lactone bound to Ile50 and Ile150, displacing the critical flap water molecule 301 found in many X-ray structures of HIV PR bound to inhibitors. The α-ethyl and α-phenyl groups in the C-3 position were in the approximate position of the S_1 and S_2 subsites respectively. The

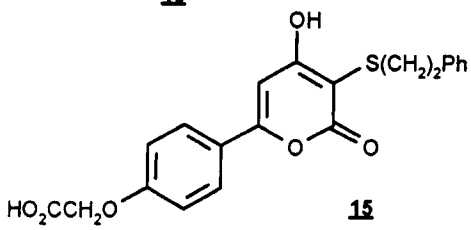
benzene ring was less effective than derivatives of the corresponding 4-hydroxypyran-2-one for accessing the S_1' and S_2' pockets. Using the X-ray structure of **13** and that of further derivatives bound to HIV PR, the potent derivative **14** was designed (HIV PR K_i = 38nM, K_i of the most active diastereoisomer = 14nM). The compound was selective with respect to other aspartic proteases and inhibited clinical isolates of HIV including AZT-resistant strains *in vitro* (IC_{50} = 3-4nM). Bioavailability is good in both rats and dogs (76% and 45% respectively), and the compound has entered clinical trials (31).



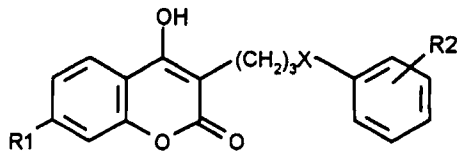
13



14

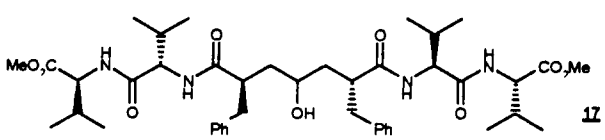


15

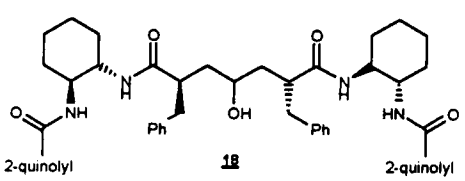


16

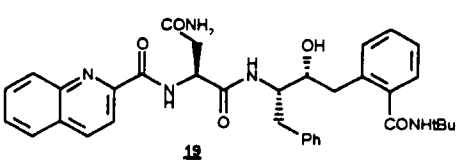
Other 4-hydroxybenzopyran-2-one (**15**) and 4-hydroxypyran-2-one (**16**) derivatives were identified and the structure-activity relationships for the series were investigated (32,33). Modelling studies on the peptidyl lead compound (**17**) indicated that the Val-Val dipeptide could be replaced with a cyclohexylamido group. Optimization of the amide substituent identified the 2-quinolyl derivative (**18**) as the most potent (HIV PR IC_{50} = 40 nM, HIV IC_{50} = 2 mM) (34). Replacement of the Phe-Pro substrate sequence with a non-hydrolysable mimic yielded nanomolar HIV PR inhibitors (**19**) which retained activity in cellular assays (HIV PR IC_{50} = 1.5nM; HIV IC_{50} = 23nM). The ortho-substituted benzamide was an effective achiral replacement for the proline residue.



17



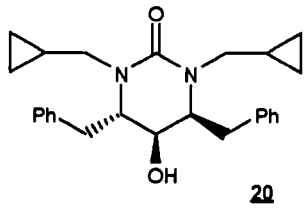
18



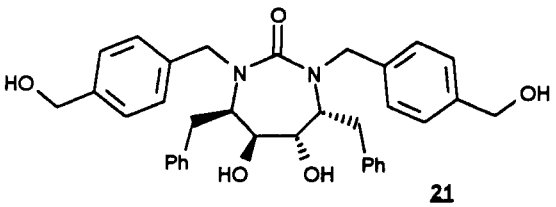
19

Crystal structures of pseudosymmetric linear peptidomimetic inhibitors bound to HIV PR show that important interactions arise from H-bonding of the amide nitrogens with structural water, interactions between the diol system and catalytic aspartates, and phenyl group binding into the S_1 and S_1' pockets. With this information, two groups independently designed 6- and 7-membered cyclic ureas (35,36). Modelling

suggested that the urea carbonyl would displace structural water and interact with the HIV PR flap, the mono-ol or diol systems would interact with the catalytic aspartates, and the phenyl groups would occupy the S_1 and S_1' pockets. The urea nitrogens readily allowed synthesis of derivatives to explore the requirements of the S_2 and S_2' pockets. Each series was optimised to afford compounds **20** and **21**, however the 7-membered ring series is 1000-fold more potent, probably due to increased binding of the diol system with the aspartates compared with the mono-ol system. Compound **21** exhibits acceptable pharmacokinetics in the rat and dog but has been withdrawn from clinical testing due to poor bioavailability.



20



21

Hepatitis C Virus Protease - Recently investigation of the properties and substrate specificities of the hepatitis C virus (HCV) serine protease has furnished useful information for inhibitor design. The HCV serine protease is homologous with trypsin-type mammalian proteases.

HCV is related to both flaviviruses (e.g. yellow fever virus) and pestiviruses (e.g. hog cholera virus). During replication, the positive sense, single-stranded RNA genome (9.5kb) is translated to produce a 3010-3033 amino acid precursor polyprotein. This polyprotein is cleaved into mature proteins by cellular and HCV-encoded proteases. Cleavage of the polyprotein in the non-structural region is catalysed by the HCV protease, itself encoded within the NS3 domain.

The catalytic triad in NS3, comprising Ser^{1165}, His^{1085} and Asp^{1107}, aligns with the catalytic triads of yellow fever and dengue virus proteases. Both Ser^{1165} and His^{1085} are essential for proteolytic activity and the protease itself is essential for processing at 4 polyprotein cleavage sites (37). HCV protease, expressed in *E. coli* as a 181-residue N-terminal fragment of NS3, is able to catalyse cleavage in *trans* at all 4 sites and in *cis* at the NS3 C-terminus (38,39). A fragment of the NS4 protein is required as a co-factor for proteolysis at 3 of the above 4 sites, and apparently acts by anchoring the protease to microsomal membranes (40).

Comparison among several HCV strains of the amino acid residues flanking the polyprotein cleavage sites suggests a consensus sequence specificity of Asp/Glu-X-X-X-X-Cys/Thr↓Ser/Ala for the HCV protease (41). Where Asp/Glu is in the P6 position relative to the scissile bond, Cys/Thr is in the P1 position, Ser/Ala is in P1', and X is any amino acid. Site-directed mutagenesis of these conserved residues in the NS3/NS4A cleavage site showed that the P6 position is relatively unimportant for defining specificity, since Asp, Asn, Lys and Ser were all acceptable. In the P1 position, amino acids with polar or aliphatic side chains were acceptable, but not amino acids with basic or aromatic side chains. In P1', basic, polar and aliphatic side chains were acceptable for proteolysis (41). Thus, the P1 position appears more restrictive than the P6 and P1' positions. Similar trends were seen for the other NS cleavage sites. The acidic residue found at P6 of all four cleavage sites was not essential for cleavage.

The HCV protease S1 specificity pocket was modeled from known structures of trypsin-like serine proteases and the conservation pattern of proteases from different HCV strains (42). This model correctly predicted that the preferred protease substrate should have a Cys at P1.

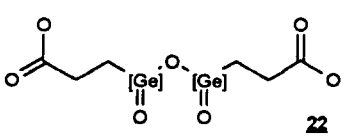
Herpesvirus Proteases - Herpesvirus proteases are essential for virion assembly in nuclei of infected cells. Herpesvirus serine proteases are homologous with each other, suggesting the possibility of discovering broad-spectrum inhibitors, but are not homologous with either mammalian (chymotrypsin) or bacterial (subtilisin) serine proteases. Site-directed mutagenesis identified Ser^{132} and His^{63} as essential amino acids in the catalytic triad of the human cytomegalovirus (HCMV) serine protease (43). EIS/MS analysis of diisopropyl fluorophosphate bound to herpes simplex virus (HSV) and HCMV serine proteases identified HCMV protease residue Ser^{132} and its HSV homologue Ser^{129} as active site nucleophiles (44,45).

Point mutations of the two HSV-1 natural cleavage sites - termed "maturation" and "release" - show that substrate specificity is localised to the P4-P1' positions (46). These data agree with cleavage site specifities predicted from sequence homologies. Similarly, simian CMV mutations of the conserved Val at P3 to Ala in the maturation site, and Tyr at P4 to Ala in the release site, virtually eliminated cleavage (43).

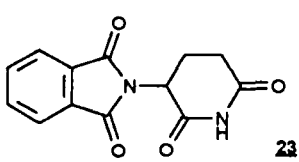
Analysis of peptides corresponding to the HCMV maturation site showed that amino acids spanning the P4 to P4' positions (VVNA/SCAL) are the minimum required for efficient cleavage (47). Conserved hydrophobic residues at P3 and P4 are essential. The rate of cleavage of a peptide spanning the release site P5-P5' (SYVKA/SVSPE) was ~100-fold lower than the rate of cleavage of a peptide spanning P5-P5' of the maturation site (GVVNA/SCRLA). K_m values for these peptides are similar, however the k_{cat} for the release site is ~8-fold lower. Peptides representing HSV-1 cleavage sites were also cleaved by the HCMV protease, but more slowly (48). In contrast, the minimum substrate corresponding to the HSV-1 maturation site is the P5-P8' peptide ALVNA/SSAAHVDV (49). This peptide was cleaved with a similar efficiency to that of the P6-P7' peptide corresponding to the HSV-1 release site (50).

IMMUNOTHERAPY OF VIRAL DISEASES

The immune system has an important role in pathogenesis and/or persistence of many viral diseases, and modulation of immune function is likely to be useful for controlling such diseases. Inhibition of virus replication by itself may not always achieve the best clinical outcome, and pharmacological induction of an appropriate immune response may be superior to antiviral therapy or a useful adjunct. Existing immunotherapies comprise vaccines, passive immunotherapy with γ-globulins, or natural cytokines such as interferon-α. However development of synthetic immunomodulators with greater antigenic specificity and better pharmacological properties is now occurring.



22



23

Chronic or persistent viral diseases such as those caused by hepatitis B virus (HBV), HCV, HIV, HSV, and CMV are the primary targets for such work. However, acute viral diseases with a significant immunological component, such as

influenza and other respiratory infections, may also benefit from an immunotherapeutic approach.

A primary objective for many chronic viral diseases is to induce viral antigen-specific cytotoxic T cells (CTL) to destroy virus-infected cells. One promising approach to this problem involves synthetic peptides which both bind MHC class 1 molecules and exhibit a virus-specific epitope. Initial results with a peptide containing a hepatitis B viral epitope indicate that it is well-tolerated and induces the desired response (51,52).

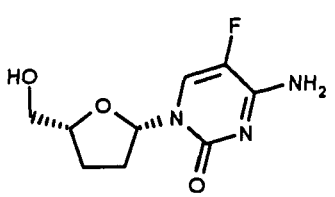
Propagermanium **(22)** was recently licensed in Japan for treatment of chronic hepatitis B. This compound induces viral antigen-specific CTL, and also increases interleukin-2 and interferon-γ secretion, but does not exhibit specific antiviral activity (53,54). The molecular target of propagermanium is otherwise unknown.

Thalidomide (**23**) has been tested clinically in AIDS patients and provides some clinical benefit (55-57). The mechanism of action for this indication has not been established, but data suggest that thalildomide may inhibit tissue necrosis factor alpha (TNF-α) function or direct the immune response towards TH-1. Both optical isomers have these effects, in contrast to the well-known teratogenicity of thalidomide which is linked to the D-isomer, thus synthesis of safer analogues may be feasible.

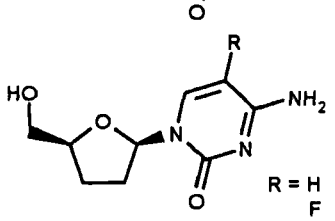
In some chronic viral diseases, AIDS particularly, reactive oxygen intermediates enhance viral replication (58, 59). A number of antioxidants are being investigated for use in AIDS. These compounds appear to direct the immune response towards TH-1, thus enhancing the cytotoxic T cell (CTL) response believed important in these conditions. For example, N-acetyl-L-cysteine increases intracellular reduced glutathione levels and exhibits moderate anti-HIV activity *in vitro* (60). The antioxidant pyrrolidine dithiocarbamate modulates HIV-1 LTR activity, but the effect is inconsistent and dependent upon the presence of phorbol esters (61). Finally, modelling studies suggest that HIV may encode selenoproteins which contribute to selenium depletion in AIDS patients (62). Clinical evaluation of antioxidants in AIDS will indicate the importance of these findings.

NEW ANTIVIRAL AGENTS

Inhibitors of HIV reverse transcriptase (HIV RT) can be divided between nucleosides and non-nucleosides. The three dideoxynucleosides analogues licensed for clinical use (AZT, ddI and ddC) have serious side-effects and, like nonnucleosides, tend to select drug-resistant virus (63). However, some nucleoside analogues with the unnatural L-configuration in the sugar moiety are as potent inhibitors of HIV RT and HIV replication as their D-configured counterparts, but are weaker inhibitors of host cell DNA synthesis.



24



R = H **25**
F **26**

2',3'-Dideoxy-β-L-5-fluorocytidine (β-L-FddC; **24**) is several-fold more active against HIV *in vitro* than 2',3'-dideoxy-β-D-cytidine (ddC; **25**) and 2',3'-dideoxy-β-D-5-fluorocytidine (β-D-FddC; **26**) with ED_{50} values of 0.5, 1.5, and 2μM respectively (64). The dose-limiting

toxicity of ddC is often neuropathy which may be associated with inhibition of mitochondrial DNA synthesis (IC_{50} = 0.022μM). Conversely, the IC_{50} values of β–L-FddC and β-L-ddC for this effect are >100μM, therefore neuropathy may not present a problem with these agents.

Nucleoside analogues require metabolism to their corresponding 5'-triphosphates to inhibit HIV RT. Some nucleoside analogue 5'-triphosphates inhibit HIV RT, but the corresponding nucleosides do not inhibit HIV replication *in vitro* due to lack of phosphorylation in the host cells. A number of approaches are being taken to bypass the need for activation by nucleoside kinases (65-67). The increased structural freedom in drug design which this allows may have implications for dealing with the emergence of resistance and may stimulate the discovery of improved therapeutic agents.

Non-nucleoside RT inhibitors are structurally diverse compounds but specific for HIV-1. The compounds are similar functionally and bind to a common site in the enzyme. HIV variants that are resistant to these inhibitors have been selected *in vitro* and more recently, from HIV-1-infected patients undergoing experimental therapy. These variants contain amino acid substitutions at RT positions that apparently interact directly with the inhibitors (68). To circumvent the rapid onset of resistance, combination therapy with other classes of HIV inhibitors is being investigated (69).

Emerging resistance to HIV PR and HIV RT inhibitors has increased the need to discover alternative modes of HIV inhibition. HIV integrase inserts pro-viral DNA into host cell chromosomes, an essential step in the HIV life cycle. The crystal structure of modified but functional HIV integrase has been reported and may facilitate structure-based inhibitor design (70). AZT mono-, di- and tri-phosphates have been shown to inhibit HIV integrase (71). Investigation of HIV vaccines continues and recent progress has been reviewed (72).

HSV-1 resistance to acyclovir often occurs through mutation of the viral thymidine kinase gene (73). Such resistance has been overcome by bypassing the mutant enzyme with the diphosphate dimyristoylglycerol prodrug of acyclovir (74). Compounds directed against two new targets for herpesviruses have been described, cyclocreatine (1-carboxymethyl-2-iminoimidazlidine) which inhibits the cellular creatine kinase/creatine phosphate system (75) and C-TRCB (76) which inhibits a virus-encoded protein required for maturation of nascent CMV DNA.

References

1. T.N. Bhat, E. Baldwin, B. Liu, Y. Cheng, J. Erickson, Nature Structural Biology 1, 552, (1994).
2. A,H. Kaplan, M. Manchester and R. Swanstrom, J.Virol, 68, 6782, (1994).
3. P.J. Bugelsi, R. Kirsh and T.K. Hart. J. Leukocyte Biology, 56,374 (1994).
4. K. Nagy, M. Young, C. Baboonian, J. Merson, P. Whittle, S Oroszlan, J. Virol., 68, 757 (1994).
5. J. Freund, R. Kellner, J. Konvalinka, V. Wolber, H.G. Krausslich and H.R. Kalbitzer, European J. Biochem., 223, 589 (1994).
6. R.B. Pollard. Pharmacotherapy, 14, 21S-29S, (1994).
7. Marketletter-February 7 (1994).
8. H. Nitschko, H. Lindhofer, H. Schatzl, J. Eberle, G. Deby, B. Kranz and K. Von der Helm. Antiviral Chem. Chemother., 5, 236, (1994).
9. A.H. Kaplan, S.F. Michael, R.S. Wehbie, M.F. Knigge, D.A. Paul, L. Everitt, D.J. Kempf, D.W. Norbeck, J.W. Erickson and R. Swanstrom. Proc. Natl. Acad. Sci. USA., 91, 5597, (1994).
10. D. D. Ho, T Toyoshima, H. Mo, D. J. Kempf, D. Norbeck, C. M. Chen, N. E. Wideburg, S. K. Burt, J.W. Erickson and M.K. Singh. J. Virol., 68, 2016, (1994).
11. M.A. el-Farrash, M. J. Kuroda, T. Kitazaki, T. Masuda, K. Kato, M. Hatanaka, S. Harada. J. Virol. 68, 233, (1994).

12. J.C. Craig, L. Whittaker, I. Duncan, N.A. Roberts. Antiviral Chem. Chemother., 5, 380, (1994).
13. E.V. Connell, M.C. Hsu and D.D. Richman. Antimicrob. Agents Chemother., 38, 348, (1994).
14. T.I. Oprea, C.L. Waller and G.R. Marshall. J. Med. Chem., 37, 2206, (1994).
15. A. Gustchina, C. Sansom, M. Prevost, J. Richelle, S.Y. Wodak, A. Wlodawer and I.T. Weber, Protein Eng., 7, 309, (1994).
16. M.R. Reddy, M.D. Varney, V. Kalish, V.N. Viswanadhan and K. Appelt. J. Med. Chem., 37, 1145, (1994).
17. J.C. Barrish, E. Gordon, M. Alam, P.F. Lin, G.S. Bisacchi, P. Chen, P.T.W. Cheng, A.W. Fritz, J.A. Greytok, M.A. Hermsmeier, W.G. Humphreys, K.A. Lis, M.A. Marella, Z. Merchant, T. Mitt, R.A. Morrison, M.T. Obermeier, J. Pluscec, M. Skoog, W. Slusarchyk, S.H. Spergel, J.M. Stevenson, C. Sun, J.E Sundeen, P. Taunk, J.A. Tino, B.M. Warrack, R.J. Colonno and R. Zahler, J. Med. Chem., 37,1758, (1994).
18. D. Scholz, A. Billich, B. Charpiot, P. Ettmayer, P. Lehr, B. Rosenwirth, E. Schreiner and H. Gstach. J. Med. Chem. 37, 3079, (1994).
19. A. Peyman, K. Wagner, K.H. Budt, J. Spanig, D. Ruppert, C. Meichsner and A. Paessens, Bioorg. Med. Chem. Lett., 4, 1191, (1994).
20. V. Van Dorsselaer, D. Schirlin, C. Tarnus, D.L. Taylor, A.S. Tyms, F. Weber, S. Baltzer, J.M. Remy, T. Brennan and D. Janowick, Bioorg Med. Chem. Lett., 4,1213, (1994).
21. T.A. Shepherd, L.N. Jungheim and A.J. Baxter, Bioorg. Med. Chem. Lett., 4, 1391, (1994).
22. S.K. Thompson, K.H. Murthy, B. Zhao, E. Winborne, D. Green, S.M. Fisher, R.L. DesJarlais, T.A. Tomaszek Jr., T.D. Meek, and J.G. Gleason, J. Med. Chem., 37, 3100, (1994).
23. B.D. Dorsey, R.B. Levin, S.L. McDaniel, J.P. Vacca, J.P Guare, P.L. Darke, J.A. Zugay, E.A. Emini, W.A. Schleif, J.C. Quintero, J.H Lin, I.-W. Chen, M.K. Holloway, P.M.D. Fitzgerald, M.G. Axel, D. Ostovic, P.S. Anderson and J.R. Huff, J. Med. Chem, 37, 3443, (1994).
24. Pink-Sheet April 18th, p 8, (1994).
25. A.K. Ghosh, H.Y. Lee, W.J. Thompson, C. Culberson, M.K. Holloway, S.P. McKee, P.M. Munson, T.T. Duong, A.M. Smith, P.L. Darke, J.A. Zugay, E.A. Emmini, W.A. Schleif, J.R. Huff and P.S. Anderson, J.Med. Chem., 37, 1177, (1994).
26. A.K. Ghosh, W.J. Thompson, P.M.D. Fitzgerald, J.C. Culberson, M.G. Axel, S.P. McKee, J.R. Huff and P.S. Anderson, J. Med. Chem, 37, 2506, (1994).
27. J.P. Vacca, P.M.D. Fitzgerald, M.K. Holloway, R.W. Hungate, K.E. Starbuck, L.J. Chen, P.L. Darke, P.S. Anderson and J.R. Huff, Bioorg. Med. Chem. Lett., 4, 499, (1994).
28. B.M. Kim, J.P. Vacca, J.P. Guare, C.M. Hanifin, S.R. Michelson, .P L. Darke, J.A. Zugay, E.A. Emini, W. Schleif, J.H. Lin, I.W Chen, K. Vastag, D. Ostovic, P.S. Anderson and J.R. Huff, Bioorg. Med. Chem. Lett., 4, 2273, (1994).
29. D.C. Humber, M.J. Bamford, R.C. Bethell, N. Cammack, D.C. Orr, R. Storer, G.G. Weingarten and P.G. Wyatt, Antiviral Chem. Chemother., 5, 197, (1994).
30. J. Kitchin, R.C. Bethell, N. Cammack, S. Dolan, D.N. Evans, S. Holman, D.S. Holmes, P McMeekin, C.L. Mo, N. Nieland, D.C. Orr, J. Saunders, B.E.V. Shenoy, I.D. Starkey and R. Storer, J. Med. Chem., 37, 3707, (1994).
31. S. Thaisrivongs, P. Tomich, K. Watenpaugh, K. Chong, W. Howe, C.P. Yang, J.W. Strohbach, S. Turner, J. McGrath, M. Bohanon, J. Lynn, A. Mulichak, P. Spinelli, R. Hinshaw, P.J. Pagano, J.B. Moon, M.J. Ruwart, K. Wilkinson, B. Rush, J. Med. Chem., 37, 3200, (1994).
32. J.V.N.V. Prasad, K.S. Para, E.A. Lunney, D.F. Ortwine, J.B. Dunbar Jr., D Ferguson, P.J. Tummino, D Hupe, B.D. Tait, J.M. Domagala, C. Humblet, T.N. Bhat, B. Liu, D.M.A. Guerin, E.T. Baldwin, J.W. Erickson and T.K. Sawyer, J. Amer. Chem. Soc., 116, 6989, (1994).
33. E.A. Lunney, S.E. Hagen, J.M. Domagala, C. Humblet, J. Kosinski, B.D. Tait, J.S. Warmus, M. Wilson, D. Ferguson, D. Hupe, P.J. Tummino, E.T. Baldwin, T.N. Bhat, B. Liu and J.W. Erickson, J. Med. Chem., 37, 2664, (1994).
34. R.E. Babine, N. Zhang, S.R. Schow, Z. Xu, R.A. Byrn, R.C. Hastings, M.F. Semmelhack, M.M. Wick and S.S. Kerwar, Bioorg. Med. Chem. Lett., 4, 583, (1994).
35. R.S. Randad, W. Pan, S.V. Gunlik, S. Burt and J.W. Erickson, Biorg. Med. Chem. Letts. 4, 1247, (1994).
36. P.Y.S. Lam, P.K. Jadhav, C.J. Eyermann, C.N. Hodge, Y. Ru, L.T. Bacheler, J.L. Meek, M.J. Otto, M.M. Rayner, Y.N. Wong, C.H. Chang, P.C. Weber, D.A. Jackson, T.R. Sharpe and S. Erickson-Viitanen. Science, 263, 380, (1994).
37. A. Grakoui, D.W. McCourt, C. Wychowiski, S.M. Feinstone, C. Rice, J. Virol. 67, 2832, (1993).
38. M.R. Eckart, M. Selby, F. Masiarz, C. Lee, K. Berger, K. Crawford, C. Kuo, G. Kuo, M. Houghton and Q.L. Choo, Biochem. Biophys. Res Comm. 192, 399, (1993).
39. C. Lin, B.M. Pragal, A. Grakoui, J. Xu and C.M. Rice, J. Virol. 68, 8147, (1994).
40. R. Bartenschlager, L. Ahlborn-Laake, J. Mous and H. Jacobsen, J. Virol. 68, 5045, (1994).

41. Y . Tanji, M. Hijikata, Y. Hirowatari and K. Shimotohno, J. Virol. 68, 8418, (1994).
42. A.A. Kolykhalov, E.V. Agapov and C.M. Rice, J. Virol. 68, 7525, (1994).
43. Y. Komoda, M. Hijikata, Y. Tanji, Y. Hirowatari, H. Mizushima, K. Kimura, and K. Shimotohno, Gene 145, 221, (1994).
44. E. Pizzi, A. Tramontano, L. Tomei, N. LaMonica, C. Failla, M. Sardana, T. Wood and R. DeFrancesco, Proc. Nat. Acad. Sci. USA 91, 888, (1994).
45. A.R. Welch, L.M. McNally, M.R.T. Hall and W. Gibson, J. Virol. 67, 7360, (1993).
46. C.L. Dilanni, J.T. Stevens, M. Bolgar, D.R. O'Boyle II, S.P. Weinheimer and R.J. Colonno, J. Biol. Chem. 269, 12672, (1994).
47. B.C. Holwerda, A.J. Wittwer, K.L. Duffin, C. Smith, M.V. Toth, L.S. Carr, R.C. Wiegand and M.L. Bryant, J. Biol. Chem. 269, 25911, (1994).
48. P.J. McCann III, D.R. O'Boyle II and Deckman, I.C., J. Virol. 68, 526, (1994).
49. V.V. Sardana, J.A. Wolfgang, C.A. Veloski, W.J. Long, K. LeGrow, B. Wolanski, E.A. Emini and R.L. LaFemina, J. Biol. Chem. 269, 14337, (1994).
50. P.J. Burck, D.H. Berg, T.P. Luk, L.M. Sassmannshausen, M. Wakulchik, D.P. Smith, H.M. Shiung, G.W. Becker, W. Gibson and E.C. Villareal, J. Virol. 68, 2937, (1994).
51. A. Sette, J. Sidney, M.-F. Del Guercio, S. Southwood, J. Ruppert, C. Dahlberg, H.M. Grey and R.T. Kubo, Molec. Immunol. 31, 813, (1994).
52. R.W. Chesnut, A. Vitiello, G. Ishioka, J.M. Grey, A. Sette and R. Rose, Abstr. Interscience Conf. Antimicrob. Agents Chemother. 34, 103 (1994).
53. Y. Ishiwata, E. Suzuki, S. Yokochi, T. Otsuka, F. Tasaka, H. Usuda and T. Mitani, Arzneimittelforschung 44, 357, (1994).
54. Y. Ishiwata, E. Suzuki, T. Otsuka, S. Yokochi, F. Tasaka, H. Usuda and T. Mitani, Pharmacometrics 47, 155, (1994).
55. P. Eggiman, J.P. Chave, M.P. Glauser and Swiss Group for Clinical Studies on Aids, Abstr. Interscience Conf. Antimicrob. Agents Chemother. 34, 199 (1994).
56. G. Reyes-Teran, J.G. Sierra-Madero, V. Martinez Del Cerro, T. Munoz-Trejo, H. Arroyo-Figueroa, A. Pasquetti, J.J. Calva, G.M. Ruiz-Palacios, Tenth Int'l Conf. AIDS 2, 65 (1994).
57. S.C. Ball, K.A. Sepkowitz, J.L. Jacobs, Tenth Int'l Conf. AIDS, Int'l Conf. STD 1, 224 (1994).
58. A.A. Colombo, E.A. Ranheim, T.C. Fong and T.J. Kipps, Blood 84 Suppl. 1, 511A (1994).
59. L.A. Herzenberg, M.T. Anderson, P. Katsikis, H. Nakamura, M. Roederer and L.A. Herzenberg, AIDS Res. & Human Retroviruses 10 (Suppl.3), S109 (1994).
60. P.A. Raju, L.A. Herzenberg, L.A. Herzenberg and M. Roederer, AIDS Res. Human Retroviruses 10, 961, (1994).
61. K. Watanabe, U. Venkatachalam and S.C. Miller, Molec. Biol. Cell 5 (Suppl), 13A (1994).
62. E.W. Taylor, C.S. Ramanathan, R.K. Jalluri, R. Nadimpalli, J. Med. Chem. 37, 2637, (1994).
63. B.A. Larder, J. Gen. Virol., 75, 951 (1994).
64. T.S. Lin, M.Z. Luo, M.C. Liu, S.B. Pai, G.E. Dutschman and Y.C. Cheng, J. Med. Chem., 37, 798, (1994).
65. C. McGuigan, P. Bellevergue, H. Sheeka, N. Mahmood and A.J. Hay, FEBS. Lett., 351, 11, (1994).
66. C. Perigaud, A.M. Aubertin, S. Benzaria, H. Pelicano, J.L. Girardet, G. Maury, G. Gosselin, A. Kirn and J.L. Imbach, Biochem. Pharmacol., 48, 11, (1994).
67. G.M. van Wijk, K.Y. Hostetler, E. Kroneman, D.D. Richman, C.N. Sridhar, R. Kumar and H. van den Bosch, Chem. Phys. Lipids, 70, 213, (1994).
68. J. Balzarini, A. Karlsson, V.V. Sardana, E.A. Emini, M.J. Camarasa and E. De Clercq, Proc. Natl. Acad. Sci. U.S.A., 91, 6599, (1994).
69. M.D. de Jong, M. Loewenthal, C.A. Boucher, I. van der Ende, D. Hall, P. Schipper, A. Imrie, H.M. Weigel, R.H. Kauffmann and R. Koster, J. Infect. Dis., 169, 1346, (1994).
70. F. Dyda, A.B. Hickman, T.M. Jenkins, A. Engleman, R. Craigie and D.R. Davies, Science, 266, 1981, (1994).
71. A. Mazumder, D. Cooney, R. Agbaria, M. Gupta and Y. Pommier, Proc. Natl. Acad. Sci. U.S.A., 91, 5771, (1994).
72. D.F. Hoth, D. Bolognesi, L. Corey, D. Vermund, Annals of Internal Medicine 121, 603, (1994).
73. D.M. Coen, Trends in Microbiology 2, 481, (1994).
74. K.Y. Hostetler, S. Parker, C.N. Sridhar, M.J. Martin, J.L. Li, L.M. Stuhmiller, G.M. van Wij, H. van den Bosch, M.F. Gardner and K.A. Aldern, Proc. Natl. Acad. Sci. USA 90, 11835, (1993).
75. J.W. Lillie, D.F. Smee, J.H. Huffman, L.J. Hansen, R.W. Sidwell and R. Kaddurah-Daouk, Antiviral Res. 23, 203, (1994).
76. R.V. Devivar, E. Kawashima, G.R. Revankar, J.M. Breitenbach, E.D. Kreske, J.C. Drach and L.B. Townsend, J. Med. Chem. 37, 2942 (1994).

Chapter 16. Therapy of *Helicobacter pylori* Infections: Current Status and Future Directions

Larry L. Klein and S. Ken Tanaka.
Anti-Infective Research, Abbott Laboratories
Abbott Park, IL 60064

Introduction - *Helicobacter pylori* (previously *Campylobacter pyloridis, C. pylori*) has been implicated as a cause of, or contributing factor in, several diseases of the human gastrointestinal tract. This spectrum of diseases includes gastritis, gastric and duodenal ulcers, and gastric cancer. Much of the evidence, especially for a causal relationship of *H. pylori* to gastritis and peptic ulcer disease is based upon the ability to observe and/or isolate the organism from involved tissues and the efficacy of therapies based on eradication of the organism. It has become apparent that antimicrobial therapy in addition to anti-secretory therapy is effective in eliminating the organism from involved tissue, promoting ulcer healing, and frequently preventing relapse. However, it has also become clear that additional or novel approaches to therapy directed toward *H. pylori* and ulcer healing are needed.

Historical Perspective - In 1984, Marshall and Warren (1) reported the isolation of a curved, gram negative, fastidious bacillus from gastric mucosa. The authors suggested that this organism, *Helicobacter pylori*, was causally related to human gastrointestinal disease. Since this initial discovery many investigators have confirmed the observation and offered extensions to other human diseases (2-6). Approximately 50% of the world population harbors this organism, and its prevalence is increased in developing countries and also in aging population (7). *H. pylori* has been identified in over 90% of duodenal ulcer patients and in 80-90% of individuals with non-specific gastritis (8). These findings will have a major impact on the use of marketed anti-ulcer drugs such as H-2 receptor antagonists and the newer H/K-ATPase inhibitors. Presently these agents account for 75% of the US pharmaceutical market (9).

In 1994, the National Institutes of Health Consensus Conference on *Helicobacter pylori*, having reviewed the available information, published a report implicating *H. pylori* in peptic ulcer disease and gastritis and supported the association with other conditions (10). This report suggests that all patients of non-autoimmune peptic ulcer disease who exhibit *H. pylori* infection "require treatment with antimicrobial agents in addition to antisecretory drugs" not only for initial or recurring disease but also in a maintenance setting.

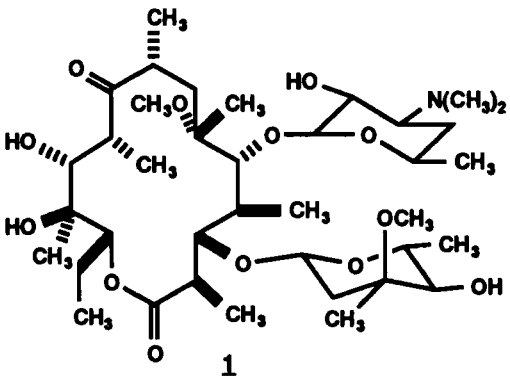
Although half the world population carries *H. pylori*, not all exhibit peptic ulcer disease; therefore, it is believed that *H. pylori* infection is a necessary but insufficient etiological agent for disease. Other factors which may contribute to this process can be related to *H. pylori* such as the high localized concentrations of ammonia, which are known to have a dose-dependent toxicity effect on the proximal cells. Other factors produced by *H. pylori* such as a 120kD cytotoxin (11-13) shown to cause cell vacuolation or the production of ulcerogenic substances such as PAF during the infection process can cause mucosal damage (14). *H. pylori* also produces large amounts of urease which may be an important virulence factor. The release of gastrin accompanies *H. pylori* infection, causing an increase in acid secretion and can induce gastric metaplasia (15). Independent factors such as smoking or stress may lead to conditions favorable for infection.

While the Consensus Conference was unable to definitively link *H. pylori* to gastric cancer, a significant relationship between infection and gastric cancer exists since *H. pylori* has been identified in 90% of gastric cancer cases (16,17). A prospective study

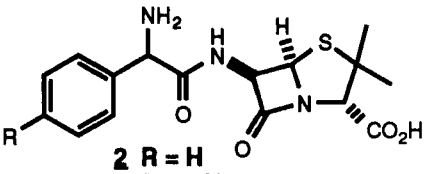
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

determined that serologic evidence of *H. pylori* infection was positively correlated with the development of gastric cancer whereas no association could be made between infection and cancer of other types (18).

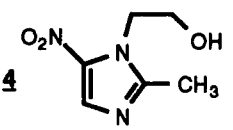
Microbiology of *H.pylori.* - *H. pylori* is an aerobic or microaerophilic curved gram negative rod. *H. pylori* is difficult to culture *in vitro*, requiring an atmosphere with increased CO_2 and media supplemented with blood or blood products. The fastidious nature of this organism has made both diagnosis of *H. pylori* infection difficult and cultures of gastric biopsies somewhat unreliable (19,20). *H. pylori* has been shown to be susceptible to several antibacterial agents under consideration as treatment modalities (Table 1). Several investigators have shown that clarithromycin (1), ampicillin (2), metronidazole (4), and tetracycline are quite active against sensitive strains *in vitro*. Ampicillin and clarithromycin have been the most active compounds to date.



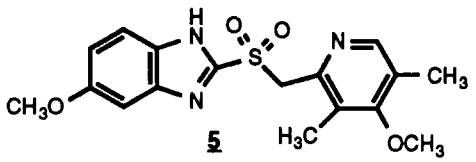
1



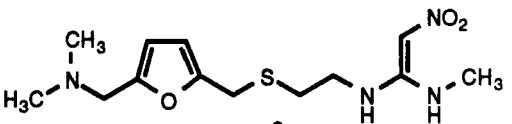
2 R = H
3 R = OH



4



5



6

The bactericidal activity of most agents appears to be related to the rate of multiplication *in vitro*. Studies have shown that ampicillin and metronidazole exhibit bactericidal activity only when cultures begin active cell division. Similar dependence on growth was observed for omeprazole (5) and bismuth (25). In contrast, clarithromycin exhibits unusual and rapid bactericidal activity against *H. pylori* which appears to be a growth independent phenomenon. One group demonstrated that the antibacterial activity of clarithromycin was due to binding to ribosomes in *H. pylori* with residence time exceedingly longer than observed for similar binding in most other organisms (26). The authors suggested that this prolonged "on-time" would result in prolonged inhibition of protein synthesis which might account for bactericidal effects.

Table 1

Antimicrobial Susceptibilities of *Helicobacter pylori*

	MIC (µg/ml)			
Agent	Range	MIC50	MIC90	Ref.
Ampicillin 2	≤0.015->0.125	≤0.015	0.031	21
Amoxicillin 3	0.015-0.25	0.06	0.12	22
	<0.016-0.06	<0.016	0.016	23
Metronidazole 4	≤0.015->128	1.0	64	21
	0.4-4	0.5	4	22
	0.064->32	1	>32	23
Tetracycline	0.06-0.25	0.12	0.12	22
	<0.016-0.19	0.05	0.125	23
Clarithromycin 1	≤0.015-0.5	0.031	0.062	21
	<0.015->4	0.015	0.03	24
Azithromycin	0.25->4	0.25	0.25	24
Ranitidine 6	3200-12800	6400	12800	22
Bismuth	2-16	8	16	22

Resistance to antibacterial agents in treatment regimens has been associated with clinical failures and recurrences. *H. pylori* resistance to metronidazole has been reported in Europe and the United States. The European Study Group on Antibiotic Susceptibility of *Helicobacter pylori* reported that primary resistance among *H. pylori* isolates occurred at a rate of 27.5% in Europe (27). Resistance to clarithromycin and other macrolides has also been reported but much less frequently. Primary resistance to clarithromycin appears to be variable since one study reported that primary resistance was about 12% in clinical isolates prior to initiation of treatment (28) and another reported 7% (29). Primary resistance to ampicillin has not been reported. Failure of treatment or recurrence of disease due to the appearance of resistant organisms has also been reported for metronidazole and clarithromycin. Isolation of metronidazole resistant *H. pylori* was associated with prior exposure of patients to metronidazole (27). The isolation of resistant *H. pylori* from 21% of patients treated with clarithromycin as the only antibacterial agent suggested selection of resistant mutants (28). The mechanisms of resistance to metronidazole and clarithromycin are not known. Ampicillin and amoxicillin (3) resistance has not been reported although *in vitro* laboratory results suggest that such mutations will be encountered in the future (30).

Clinical Therapeutic Experience - There have been few reports of large, well controlled clinical evaluations of the various anti-*H. pylori* regimens described in the literature; however, many small therapeutic trials, controlled and uncontrolled, have been reported. There have been few monotherapy trials of antibacterial agents alone; most include anti-ulcer medication such as an antacid preparation (bismuth salt), H2 antagonist, or proton pump inhibitor. In general these trials have shown that single agent therapy results in less than half the patients exhibiting favorable response, although those with demonstrated eradication of *H. pylori* are less subject to recurrence in long-term followup (31). For example, one study reported that clarithromycin monotherapy resulted in eradication of *H. pylori* in 42% of patients (32) and another reported eradication in 54% of cases (28).

Generally, dual or triple therapies have been the most promising regimens. These therapies typically include one anti-ulcer medication plus one or two antibacterial agents. Various combinations have been or are undergoing evaluation, including combinations of antiulcer medications (bismuth, ranitidine, or omeprazole) plus clarithromycin or amoxicillin, or a combination of antibacterial agents (clarithromycin or amoxicillin plus metronidazole, or clarithromycin or amoxicillin plus tetracycline). Therapy that included clarithromycin or amoxicillin and an antiulcer medication was also demonstrated to be effective (29, 34). Triple therapies that included an antiulcer medication with two antibacterial agents were also highly effective (35-39). Unfortunately problems associated with side-effects, resistance (25% *H. pylori* strains in US resistant to metronidazole (33)) and patient compliance with the complicated and numerous daily dosing schedule of these three unrelated agents have occured.

The results of these clinical evaluations indicate the importance of antibacterial therapy in ulcer healing and, perhaps more importantly, they suggest that eradication of *H. pylori* prevents recurrence of disease. Of the patients in whom *H. pylori* was not eradicated but for whom ulcer healing was evident, 86% relapsed within 12 months after cessation of therapy (36). This was in contrast to 8% of patients relapsing after demonstrated eradication at the end of therapy.

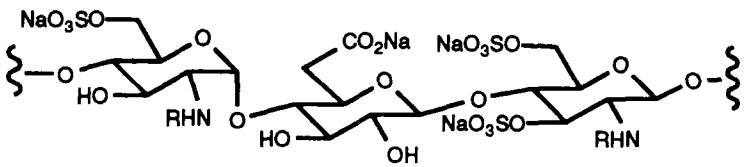
The therapeutic trials of antibacterial and antiulcer medications raise several important issues that need to be addressed in the development of new treatment modalities. It is clear that new therapies for peptic ulcer disease and *H. pylori* must eliminate the organism from lesions in order to prevent recurrence or relapse. There is evidence that at least in the case of ampicillin, the antimicrobial efficacy may be due to systemic delivery of the antibiotic to the diseased tissue rather than topical delivery by diffusion through the gastric mucus (40). Whether this systemic component is important in all cases and for all antimicrobials remains to be proven.

It is also important to note that while several of the antibacterial agents are extremely potent, rapidly bactericidal, and attain high tissue concentrations in experimental animals (41), the level of therapeutic efficacy suggests that other factors might impact the ability of antibacterial agents to eradicate the organism. Although clarithromycin is one of the most active antibacterial agents against *H. pylori*, there is clearly therapeutic benefit to the inclusion of antiulcer medication to the treatment regimen. This may reflect local microenvironmental factors in diseased tissue that prevent clarithromycin (and other antibacterial agents) from exerting their full antimicrobial activity. A better understanding of these factors should facilitate the evaluation and design of future antibacterial agents for the treatment of ulcer disease.

FUTURE DIRECTIONS

H. pylori Adhesion Blockers - An important step in the pathogenesis of *H. pylori* infection is adhesion of the organism in the gastric mucosa. Inhibition of its specific binding to contact sites located on the gastric epithelial cells serves as a potential

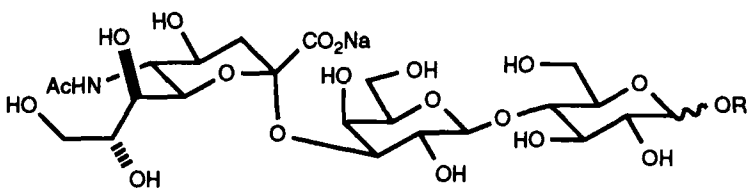
target for anti-*H. pylori* therapy. Furthermore, since the turnover rate for gastric epithelial cells is less than one day, the organism must undergo multiple attachment processes, each event offering the potential for interference by exogenous adhesion blockers. Inhibition of the adherence of *H. pylori* to the gastrointestinal tract thus halts colonization and, when treated in conjunction with an antibiotic or an anti-ulcer drug such as ranitidine or omeprazole, would promote eradication. Recently, studies of appropriate models for this binding event have shown strong binding to exist between *H. pylori* and a number of macromolecules such as plasminogen and vitronectin (42), and also human mucin, a major glycoprotein of the gastric mucosal layer. Structure-binding experiments have begun to uncover the functional/structural requirements for binding to the organism. For example, *H. pylori* exhibits strong binding to some charged carbohydrate oligomers such as heparin (segment shown as **7**), heparan sulfate and dextran sulfate, though not with the corresponding neutral saccharides (43).



7

One study utilizing HEp-2 cells as models for this binding identified a sulphated glycerolipid found in mammalian stomach tissues as a potential adhesion site (44). A related approach proposed the use of a different sulfated oligosaccharide which is immobilized (covalently linked) to a support such as Sephadex in order to allow for purification of the specific surface binding protein of *H. pylori*. Further advantage of these carbohydrate adhesion agents as potential therapeutic or diagnostic tools can be gained by linking to an insoluble carrier such as chitin (43).

Characterization of the adhesion molecules in some model systems has shown sialyl-based residues to be implicated. Molecules present on the surface of *H. pylori* thus may interact with N-acetyl-neuraminic acid (sialic acid) terminated molecules present on gastric epithelial cells. For example, the binding of *H. pylori* to model erythrocyte receptors was inhibited by sialosaccharides, and it was proposed that a particular structural unit, NeuAc(2-3)lactose, may be the key molecular fragment which binds to these sites on *H. pylori* (45). Based on these and other findings, one patent proposes the use of a specific set of sialyl-containing oligosaccharides such as typified by **8** as anti-*H. pylori* agents (46).



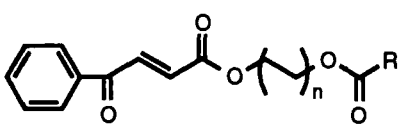
8 **R = H or aminoacid**

Urease Inhibitors - Few organisms can exist in the harsh environment present in the gastric cavity. It has been suggested that one of the reasons *H. pylori* can survive and flourish in the stomach involves its utilization of a urease enzyme which acts to cleave urea into ammonia and carbon dioxide thus increasing the pH in its proximity. The urease enzyme in *H. pylori* accounts for 5-10% of its total protein and has been shown to be one of the most powerful such enzyme in any organism (47). Urease deficient *H. pylori* strains were unable to colonize in the gnotobiotic piglet model even

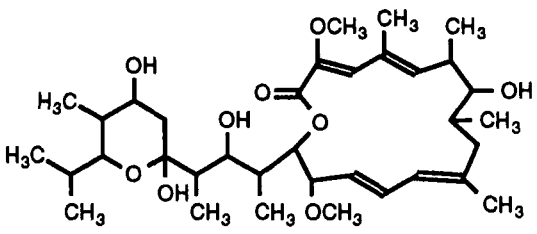
in the presence of urease-positive bacteria. These studies have shown that not only the presence of this enzyme, but its ammonia-producing action is critical for *H. pylori* colonization (48). The inhibition of this activity may be exploited as a viable target.

Several approaches described in patents have not pursued small molecule inhibitors but rather an immunologic approach via the use of antigens of the urease enzyme or portions thereof. Any such approach targeting urease antigens would have to use high doses in order to counteract the strong antibody response, depend on oral administration in order to provide access to the organism and avoid the expected epithelial cell toxicity. Crude lysates of the *Helicobacter* organisms have been used to induce activation of the mucosal immune system to produce anti-urease antibodies. Although *in vivo* models of *H. pylori* infections are rare, one group generated antibodies by utilizing a model of a related organism, *Helicobacter felis* in germ-free mice (49). The method took advantage of the cross reactivities of *H. felis* and *H. pylori*. This group found that the urease enzyme is actually composed of two subunits only one of which gave rise to a protective response. When oral immunization of the mice took place, the animals exhibited a reduced infection rate such that 70% of animals were protected 12 days post challenge compared to no protection from the alternate subunit. They have thus identified a urease antigen of *H. pylori* as a potential vaccine and showed efficacy in an animal model.

Other Approaches - A bactericidal compound known as ambicin has been used through the years as a food preservative and has shown anti-*H. pylori* activity (50). Due to its use in the food industry, safety issues have already been addressed. Another patent describes a set of benzoylacrylic esters **9** which are shown to exhibit antibacterial activity (MIC = 0.9 mcg/mL) when tested against *H. pylori* cultures isolated from various ulcers of human patients (51). Furthermore, these agents show activity (ED_{50} = 26mg/kg) in the standard Shay rat model assay (acid secretion model) after oral dosing implying an additional unrelated effect on the production of gastric acid. Agents which can be shown to have both antibacterial and anti-acid characteristics in animals could supercede any multidrug therapy currently available.



9 n = 0,1,2
R = alkyl, phenyl



10

The bafilomycin series of naturally occuring macrolides (e.g., bafilomycin A_1 (**10**)) originally known for their weak antibacterial and antifungal activity, has been shown to have inhibitory activity toward vacuolar-type H^+ - ATPase. Vacuolation, a process whereby cytoplasmic vacuoles are induced to absorb fluid is known to play a major role in cell death. Vacuolation of gastric tissues is believed to be induced by *H. pylori* upon infection and is related to the action of vacuolar-type H^+ - ATPases, enzymes which promote a proton gradient across cell membranes. The basilomycins have been shown to both prevent and reverse this vacuolation through an unknown mechanism and may represent an alternative approach to treatment.

Summary - The recognition and acceptance by the medical community of the role of *H. pylori* in peptic ulcer disease and its potential involvement in other gastric diseases has resulted in a reevaluation of therapies that had been targeted to the

hypersecretory component only. As a bacterial infectious disease, many therapeutic modalities now have at least one component directed toward eradication of the organism. Clinical trials have clearly shown that eradication of *H. pylori* is essential to prevent recurrance of disease that has in the past been the hallmark of anti-secretory therapies.

It has also become clear that the rather complex treatment regimens, resistance to antibacterial agents, and perhaps special characteristics of the host-parasite relationship indicate the need for novel approaches. Thus, it appears that opportunities exist to address the therapy of peptic ulcers and perhaps other gastric diseases exploiting virulence factors, such as urease, cytotoxin, or adherence factors, or via vaccines. It is likely that additional opportunities may arise from our increasing understanding of the complex issues of the host-parasite interactions that are only now becoming evident.

References

1. B.J. Marshall and J.R. Warren, Lancet, 1, 1311 (1984).
2. J. Labenz and G. Borsch, Gut, 35, 19 (1994).
3. D.Y. Graham, Gastroenterology, 96, 615 (1989).
4. M. Asaka, M. Kudo, M. Kato, T. Kimura, T. Meguro, S. Mitani, T. Miyazaki, and K. Inoue, Gastroenterology, 29 (Suppl VII), 100 (1994).
5. N. Munoz, Cancer Epidem., Biomarkers, Prevent., 3, 445 (1994).
6. S.J.O.Veldhuyzen van Zanten and P.M. Sherman, Can. Med. Assoc. J., 150, 177 (1994).
7. B. van der Linden, Curr. Opin. Infec. Dis., 7, 577 (1994).
8. T. G. Reilly, R.P. Walt, J. Clin. Pharm. Therap., 19, 73 (1994).
9. A. Garner, S.M.A. Bastaki, M.Y.Hasan, International Pharm. J., 8, 17 (1994).
10. NIH Consensus Development Panel on *Helicobacter pylori* in Peptic Ulcer Disease, J. Amer. Med. Assoc., 272, 65 (1994).
11. J.E. Crabtree, J.D. Taylor, J.D. Wyatt, R.V. Heatley, T.M. Shallcross, D.S. Tompkins, B.J. Rathbone, Lancet, 338, 332 (1991).
12. T.L. Cover and M.J. Blaser, J. Biol. Chem., 267, 10570 (1992).
13. A.T.R. Axon, J. Antimicrob. Chemother., 32, (Suppl A), 61 (1993).
14. Y. Denizot, I. Sobhani, J.C. Rambaud, M. Lewin, Y. Thomas, J. Benveniste, Gut, 31, 1242 (1990).
15. P. Malfertheiner, J.E. Dominguez-Munoz, Clin. Ther. 15(Suppl.B), 37 (1993).
16. J. Parsonnet, Eur. J. Gastroent. Hepatol., 5 (Suppl 1), S103 (1993).
17. P. Sipponen, Scand. J. Gastroenterol., 29 (Suppl 201), 24 (1994).
18. L.-E. Hansson, L. Engstrand, O. Nyren, D.J. Evans, Jr., A. Lindgren, R. Bergstrom, B. Andersson, L. Athlin, O. Bendtsen, and P. Tracz, Gastroenterology, 105, 1098 (1993).
19. J.A. Schrader, H.V. Peck, W.M. Notis, P. Shaw, and R.A. Venezia, Amer. J. Gastroenterol., 88, 1729 (1993).
20. R. Ansborg, G. Von Recklinghausen, R. Pomarius, and E.N. Schmid, J. Clin. Micro., 29, 51 (1991).
21. A.S. Weissfeld, D.E. Simmons, D. Busch, R.K. Flamm, S.K. Tanaka, A. Ciocola, and D. Webb, Amer. J. Gastroenterol. 89, 1390 (1994).
22. C.S. Goodwin, P. Blake, and E. Blincow, J. Antimicrob. Chemother., 17, 309 (1986).
23. Y. Glupczynski, M. Labbe, W. Hansen, F. Crokaert, and E. Yourassowsky, J. Clin. Micro., 29, 2072 (1991).
24. G.J. Malanoski, G.M. Eliopoulos, M.J. Ferraro, and R.C. Moellering, Jr., Eur. J. Clin. Micro. Infect. Dis., 12, 131 (1993).
25. R.K. Flamm, J. Beyer, S.K. Tanaka, and J. Clement, Second International Conference on the Macrolides, Azalides, and Streptogramins, Venice, Italy (1994).
26. R.C. Goldman, D. Zakula, R. Flamm, J. Beyer, and J. Capobianco, Antimicrob. Agents Chemother., 38, 1496 (1994).
27. European Study Group on Antibiotic Susceptibility of *Helicobacter pylori*, Eur. J. Clin. Microbiol. Infect. Dis., 11, 777 (1992).
28. W.L. Peterson, D.Y. Graham, B. Marshall, M. Blaser, R. Genta, P. Klein, C. Stratton, J. Drnec, P. Prococimer, and N. Siepman, Amer. J. Gastroenterol., 88, 1860 (1993).
29. R.P.H. Logan, P.A. Gummett, H.D. Schaufelberger, R.R.F.H. Greaves, G.M. Mendelson, M.M. Walker, P.H. Thomas, J.H. Baron, and J.J. Misiewicz, Gut, 35, 323 (1994).
30. C.E. Haas, D.E. Nix, and J.J. Schentag, Antimicrob. Agents Chemother., 34, 1637 (1990).
31. G.D. Bell and K. U. Powell, Scand. J. Gastroenterol., 28 (S196), 7 (1993).
32. D.Y. Graham, J. Drnec, and P. Prococimer, First International Conference on the Macrolides, Azalides, and Streptogramins, Santa Fe, NM (1992).

33. P. Cotton, JAMA, 271, 808 (1994).
34. J. Labenz, E. Gyenes, G.H. Ruhl, and G. Borsch, Gut, 34, 1167 (1993).
35. D.Y. Graham, Gastroenterol., 104, A90 (1993).
36. E. Hentschel, G. Brandstatter, B. Dragosics, A. Hirschl, H. Nemec, K. Schutze, M. Taufer, and H. Wurzer, New Engl. J. Med., 328, 308 (1993).
37. J.C. Thijs, A.A. Van Zwet, and H.B. Oey, Scand. J. Gastroenterol., 28, 934 (1993).
38. S.W. Hosking, T.K.W. Ling, S.C.S. Chung, A.F.B. Cheng, J.J.Y. Sung, and A.K.C. Li, Lancet, 343, 508 (1994).
39. M.T. Al-Assi, F.C. Ramirez, G.L. Lew, R.M. Genta, and D.Y. Graham, Amer. J. Gastroenterol., 89, 1203 (1994).
40. R.J. Adamek, M. Wegener, W. Opferkuch, and G.H. Ruhl, Amer. J. Gastroenterol., 88, 792 (1993).
41. K. Chang, F. Wyle, G. Skankey, J. Kidao, D. Remiggio, A. Lai, and C. Hong, Gastroenterology, 104, A680 (1993).
42. M. Ringner, K.H. Valkonen, T. Wadstrom, FEMS, 9, 29 (1994).
43. Medicarb. O. Larm, T. Wadstrom, Patent WO 9416714.
44. C.A. Lingwood, A. Pellizzari, H. Law, P. Sherman, B. Drumm, Lancet, 238 (1989).
45. D.G. Evans, D.J. Evans, J.J. Moulds, D.Y. Graham, Infect. Immun., 56, 2896 (1988).
46. Neose Pharmaceuticals, Inc. S. Roth, E. McGuire, D. Langer, WO 9403184.
47. L.-T. Hu, H.L.T. Mobley, Infect. Immun., 58, 992 (1990).
48. K.A. Eaton, S.Krakowka, Infect. Immun., 62, 3604 (1994).
49. Fondation Pour LaRecherche des Maladies Gastro-Intestinales, P. Michette, A. Blum, C. Davin, R. Haas, I. Corthesy-Theulaz, J.-P. Kraehenbuhl, E. Saraga, WO 9409823.
50. Richter Gedeon Vegyeszeti Gyar Rt. I. Ballo, J. Fischer, E. Petenyi, E. Ezer, J. Matuz, K. Saghy, L. Szpomy, G. Hajos, WO 9414749.
51. Biocine Sclavo S.P.A., M. Bugnoli, C. Montecucco, E. Papini, R. Rappuoli, WO 9404161.

SECTION IV. IMMUNOLOGY, ENDOCRINOLOGY AND METABOLIC DISEASES

Editor: William K. Hagmann
Merck Research Laboratories, Rahway, NJ 07065

Chapter 17. Recent Advancements in the Discovery and Development of Agents for the Treatment of Diabetes.

Robert L. Dow and David K. Kreutter
Central Research Division, Pfizer Inc
Groton, CT 06340

Introduction - Diabetes is a chronic disease affecting 5% of the population of the industrialized world. In the U.S. there are 14 million diabetics, 98% of which have type II or non-insulin-dependent diabetes (NIDDM). While it is clear that there is a genetic basis for NIDDM, the relative roles of genetics and environment have yet to be established. There are 1-2 million individuals with type I or insulin-dependent diabetes (IDDM). This form of the disease is due to an autoimmune response, leading to destruction of the endocrine pancreas, and therefore the requirement for exogenous insulin to sustain life. The total health care costs for NIDDM in the U.S. is estimated to be $105 billion, the majority being due to treatment of the disease-associated complications (1). The recently completed NIH-sponsored Diabetes Control and Complications Trial (DCCT) demonstrated that tight glucose control is associated with reduced incidences of the major complications: retinopathy, nephropathy and neuropathy (2). Therefore, lowering blood glucose may be an effective mechanism for preventing the development of diabetic complications.

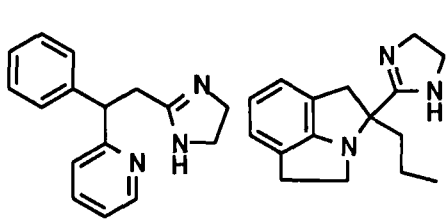
NIDDM is characterized by abnormal insulin secretion from the pancreas, increased basal glucose output by the liver and insulin resistance in peripheral tissues (3,4). In the last several years, clinical measurements of glucose disposal rates in man have made it possible to show that peripheral insulin resistance significantly contributes to the hyperglycemic state of the diabetic (3,5). Research into therapy has targeted the pancreas, liver and peripheral tissues in order to achieve glucose lowering.

Enhancers of Insulin Release - The sulfonylurea class of agents, which stimulate the release of insulin, has served as the major oral therapy for NIDDM patients for over thirty years (6, 7). It has been proposed that sulfonylureas inhibit the ATP-sensitive K^+ channels in the pancreatic B-cells leading to the efflux of potassium and a depolarization of the cell membrane. This in turn triggers voltage-dependent Ca^{2+} channels, increasing intracellular calcium and inducing exocytosis of insulin granules (8). Following the discovery of sulfonylureas, a number of other chemical entities have been identified which exhibit insulin secretatory activity (9).

A potential alternative mechanistic approach to the stimulation of insulin release is based on the finding that catecholamines reduce the rate of insulin secretion from the pancreas (10). It has subsequently been shown that this effect is mediated through the α_2-adrenoceptor (11). Significant research efforts have been devoted to the imidazoline-based class of α_2-adrenergic antagonists, represented by midaglizole (DG-5128, 1) (12-20). In NIDDM patients, midaglizole (1) improves insulin secretion and glucose disposal (21, 22). A number of recent studies raise the possibility that these imidazoline-based compounds may be exerting their effects via a mechanism(s) distinct from that of α_2-blockade (12, 13). This hypothesis arises from the finding that insulin secretagogue activity is not always well correlated with adrenergic inhibition. Many of

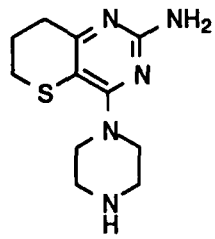
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

these agents inhibit B-cell ATP-sensitive K^+ channels and it has been proposed that there may be a novel imidazole binding site present in islets (12,13). Stereoselectivity is observed for the inhibition of α_2-adrenoceptors by the enantiomers of **2** (SL 84.0418), but not for the effects on ATP-sensitive K^+ channels, leading to the conclusion that stimulation of B-cells is mediated mainly through K^+ channels (16). Imidazoline **2** produces a dose-dependent decrease in plasma glucose following an oral glucose load in healthy humans (17, 18), an effect not observed for idazoxan an earlier member of this class (23, 24).

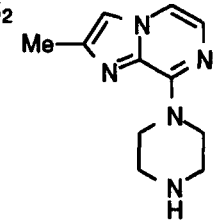


1

2

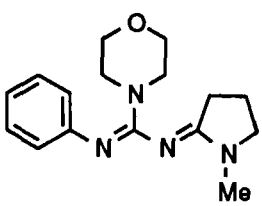


3

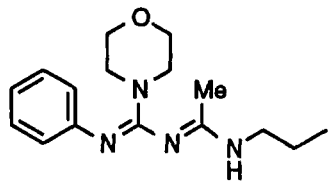


4

A series of 1-piperazinylimidazopyrazines (e.g. **4**) have been prepared (25) as a follow-up to the antihyperglycemic thiopyranopyrimidine **3** (MTP-1403, 26). Members of the heteroarylpiperazine class have been reported to possess insulin secretagogue activity (27). Compound **4** is approximately 25-fold more potent than **3** in lowering the hyperglycemic state in an obese mouse model of NIDDM and significantly (K_i = 51 nM) inhibits α_2-adrenergic binding. A structure-activity-relationship study around the clinically-efficacious, insulin secretagogue linogliride (**5**) has revealed **6** to be 6-fold more potent than **5** in the rat glucose tolerance test (28,29).



5



6

Glucagon-like Peptide-1 - Glucagon-like peptide-1(GLP-1) is a product of the proglucagon gene that is secreted from intestinal L cells and enhances insulin secretion from the pancreatic β cell in a glucose-dependent manner (30). The biologically active forms of GLP-1 are the truncated peptides, GLP-1(7-37) and GLP-1(7-36) amide, the latter being the predominant form of the hormone that is expressed (31, 32).

Structure-activity studies on GLP-1 demonstrate that the N-terminus of the peptide is important for receptor binding and activation, and that specificity for the GLP-1 receptor may reside in the C-terminus (33, 34). It appears that amino acid sequences in the C-terminal half of the molecule are less important determinants of receptor binding and insulinotropic activity than are those in the N-terminal half (35).

In normal, healthy individuals there is no difference in the incretin effect of GLP-1(7-37) and GLP-1(7-36) amide. Both peptides have similar plasma half lives and metabolic clearance rates and comparably stimulate insulin and C-peptide secretion and

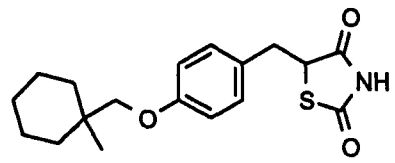
inhibit glucagon secretion (36). In NIDDM, the incretin effect of oral glucose is blunted which does not appear to be due to diminished secretion of GLP-1 (37). Basal GLP-1 levels in the circulation are elevated in NIDDM (38-40). Infusion of GLP-1(7-36) amide into subjects with NIDDM induces insulin secretion to 70% of that observed in normal subjects (40). Similar results have been observed with GLP-1(7-37) infusion; insulin secretion is stimulated 3-10 fold in both normal subjects and those with NIDDM (41). Infusion of either GLP-1(7-36) amide or GLP-1(7-37) reduces the postprandial plasma glucose excursion in subjects with NIDDM, demonstrating the potential utility of GLP-1 in the treatment of NIDDM (41, 42). In subjects with NIDDM, infusion of GLP-1(7-36) amide reduces the amount of insulin required to maintain postprandial euglycemia, suggesting that in addition to the enhancement of insulin secretion, diminution of glucagon secretion and improvement in insulin sensitivity contribute to the antidiabetic effects of GLP-1 (42). Analysis of intravenous glucose tolerance tests in normal subjects by Bergman's minimal model demonstrates that GLP-1 increases insulin-independent glucose disposal in addition to its insulinotropic action (43).

Insulin Analogs - The design of modified insulin analogs has been based on the goal of obtaining injectable insulins with pharmacokinetic profiles that approximate endogenous insulin secretion (44). Analogs have been designed for meal-related therapy that are rapidly absorbed from the site of injection in order to prevent meal-associated hyperglycemia and that have a short duration of action to prevent between meal hypoglycemia (44)

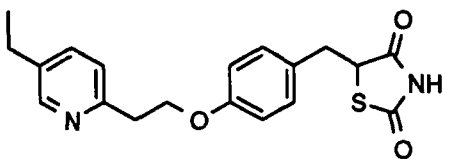
Insulin normally exists as a hexamer, which requires the presence of proline at position 28 of the B chain; removal or replacement of this residue reduces the rate of association of the insulin monomers (45). Replacement of Pro(B28) with Asp results in a fasting acting insulin analog (46). Euglycemic clamp experiments in healthy human subjects demonstrate that Asp(B28)-insulin is absorbed ~25% faster than regular insulin and has slightly reduced potency compared to regular insulin (46) Similar pharmacokinetics are observed with des-(B26-B30)-insulin-B25-amide, an analog that is truncated and amidated at the carboxy terminus. This analog is monomeric in solution, favoring rapid absorption (47). Peak serum levels in humans are observed after 30-45 minutes for the analog, compared to 60 minutes for regular insulin (48). However, there was no increase in the rate of glucose lowering with this modified insulin, despite full biological potency (49). [Lys(B28), Pro(B29)]-human insulin is an analog in which the amino acid sequence at positions 28 and 29 of the B chain are inverted in order to limit the self-association of the molecule (45). After subcutaneous injections of [Lys(B28), Pro(B29)]-insulin in humans, maximum serum concentrations are twice as high and are attained in half the time as compared to injection with regular insulin (50). During a glucose clamp, the glucose infusion rate required to maintain euglycemia peaked in half the time after subcutaneous injection of [Lys(B28), Pro(B29)]-insulin, compared to regular insulin (50). The total glucose infused was the same in both insulin treatment groups, suggesting that [Lys(B28), Pro(B29)]-insulin is a fully potent insulin analog (50). In a one year clinical trial in 631 patients with both type I and II diabetes, there was an improvement in postprandial glycemic control and no increase in hypoglycemic episodes with [Lys(B28), Pro(B29)]-insulin, compared to regular insulin (51). A similar study of 9 months duration demonstrated a decreased glycosylated hemoglobin in the [Lys(B28), Pro(B29)]-insulin treated group, compared to the regular insulin group, suggesting a therapeutic advantage for [Lys(B28), Pro(B29)]-insulin (52).

Enhancers of Insulin Action - During the past twelve years, significant research efforts have been devoted to a class of agents which enhance insulin action *in vitro* and *in vivo* without stimulating insulin secretion or producing frank hypoglycemia. The prototypical agent in this class, ciglitazone (7), lowers plasma glucose in genetic animal models of NIDDM, but has no effect in normal animals (53-55). Structure-activity-relationship studies have led to the discovery of a number of agents which have entered clinical development, including pioglitazone 8 (56-58), englitazone 9 (59,60) and troglitazone 10 (CS-045) (61,62).

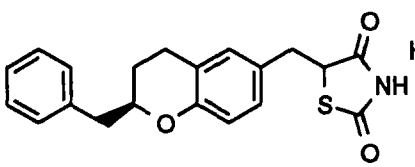
Troglitazone (10) is the most advanced member of the thiazolidinedione class of hypoglycemic agents, with an NDA submitted in Japan and advanced clinical studies underway in North America and Europe (63). Recent reports of clinical results in NIDDM and obese subjects reveal a number of parameters that are positively impacted (64-66). When NIDDM patients were treated with a six to twelve week course of 10, fasting blood glucose and area under the curve for oral glucose tolerance were significantly improved (21% and 17%, respectively) (64,65). Of the eleven patients in the latter study, eight had a marked clinical response whereas three were nonresponders (65). When the nonresponders are removed from the analysis, troglitazone's impact on various metabolic parameters is even more striking. In obese patients, with either normal or impaired glucose tolerance, 10 increases glucose disposal by 28%, decreases fasting plasma insulin by 48% and slightly decreases both systolic and diastolic blood pressure (66). These findings suggest that this agent has therapeutic potential in a subpopulation at risk for the development of NIDDM.



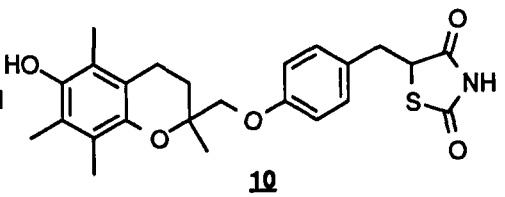
7



8



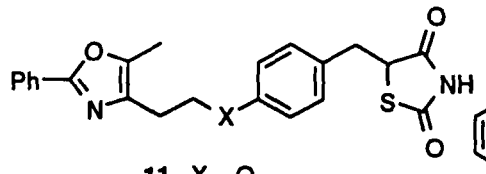
9



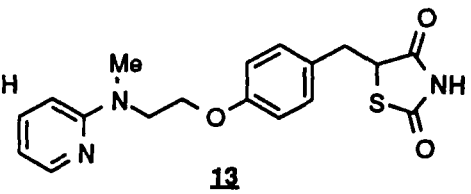
10

Given the unique biological activities and initial positive clinical effects observed for the thiazolidinediones, a significant amount of effort is being directed towards the identification of agents with enhanced oral potencies and improved side effect profiles (67-79). Replacement of the cyclohexyl portion of 7 with a phenyloxazole moiety (11, 12) improves oral hypoglycemic effects by 500-fold (67,68). Because members of this class have been observed to adversely affect bone marrow function and red cell volume, agents with improved toxicological profiles have been targeted (80,81). A N-methylamino variant (BRL-49653, 13) of 8 produces no significant reduction in blood hemoglobin content at doses 100-fold above those which produce a 25% reduction in area under the glucose tolerance curve (69,70). In the case of 10 this separation is only 1.5-fold (70). At high doses, amide (14) is reported to be devoid of significant red blood cell, hemoglobin and leukocyte lowering properties (71).

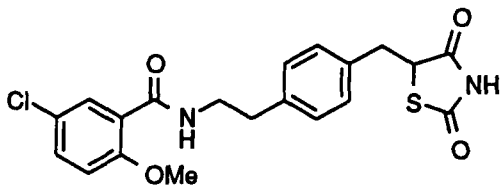
There have been a number of reports which show that hypoglycemic activity is retained for compounds in which the thiazolidinedione functionality is replaced by related acidic functionalities such as 5-oxathiadiazole-2-oxide (77), 1-oxadiazoline-2,4-dione (78) and 5-oxazolidine-2,4-dione (79). It is not known if the mechanism by which these agents reduce hyperglycemia in animal models of NIDDM is similar to that of the thiazolidinediones. An alternative mechanism may be operative for the 5-oxathiadiazole-2-oxide class (15) since it produces glucosuria in normal mice, an effect not observed for the thiazolidinediones (77).



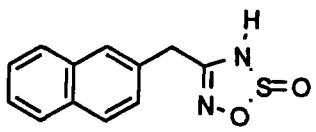
11 X = O
12 X = C(O)



13

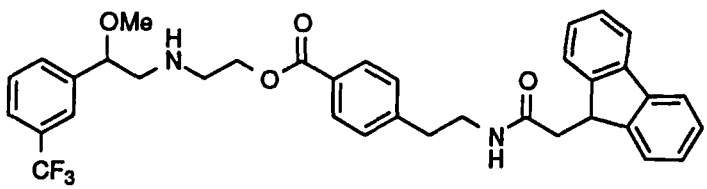


14



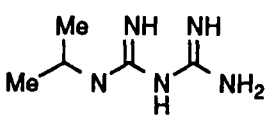
15

As part of a follow-up study on a series of lipid lowering agents, 16 (S15261) has been discovered to potently decrease plasma insulin (0.5-2.5 mg/kg, p.o., b.i.d. for 14 days) and increase disposal of glucose in obese, insulin-resistant rats (82). This compound increases peripheral sensitivity to insulin, though the molecular mechanism producing this effect is not understood. As seen for the thiazolidinedione class of hypoglycemic agents, 16 does not produce hypoglycemia in normal animals.

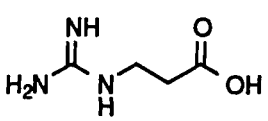


16

The glucose lowering effects of the biguanide-class (e.g. metformin, 17) in NIDDM patients is well established (83). It has been proposed that the lipophilic side chain present in these agents is responsible for the limiting clinical side effect of lactic acidosis (84,85). Reexamination of guanidines containing polar, acidic functionality, such as 3-guanidinopropionic acid (18), in more relevant models of NIDDM has reversed the previously held conclusion that these compounds are not antihyperglycemic (86). This finding may have relevance for the discovery of agents with improved side effect profiles.



17



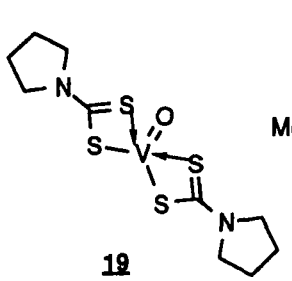
18

β_3-Adrenergic Receptor Agonists - The potential utility of β_3-adrenergic agonists in the treatment of diabetes is discussed in Chapter 21 of this volume.

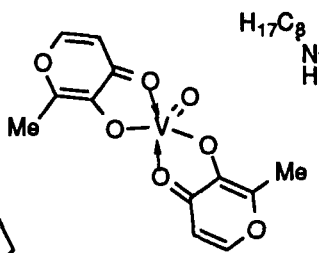
Vanadate - The insulin mimetic effect of vanadate (VO_4^{3-}, vanadium (V)) has been known since 1980 (87). *In vivo*, vanadate is reduced to vanadyl ion (VO^{2+}, vanadium (IV)) (88). Addition of sodium ortho vanadate or vanadyl sulfate to the drinking water of

insulin deficient, streptozotocin-diabetic rats normalizes plasma glucose (89-91). Treatment of streptozotocin-diabetic rats with vanadyl sulfate for one year causes sustained glucose lowering with no development of tolerance (92). Oral vanadate also decreases the hyperglycemia in the ob/ob and db/db models of type II diabetes (93,94). Supplementation of primary therapy in type II diabetic humans with vanadate for two weeks or vanadyl sulfate for three weeks improves insulin sensitivity and decreases HbA1c levels (95,96).

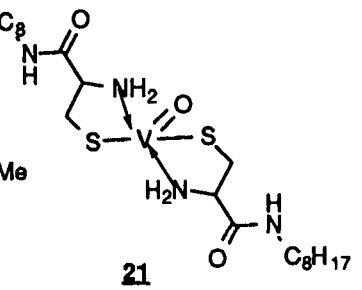
Treatment of rats with therapeutic doses of vanadate is associated with toxicity that is expressed primarily as dehydration and diarrhea (89,97). Food intake is also reduced, which has been suggested as the mechanism for the antidiabetic effects of vanadate (97). Vanadyl sulfate is less toxic than vanadate (98), but treatment of rats with vanadyl sulfate results in the accumulation of vanadium in bone, kidney, testis, liver, pancreas and brain, which persists for 16 weeks after withdrawal of vanadyl treatment (99). Efforts have been made to decrease the toxicity of vanadate by making vanadyl-sulfur complexes. Bis(pyrrolidine-N-carbodithioato)oxo-vanadium(IV) (**19**) is a potent insulin mimetic agent *in vitro*, and lowers blood glucose in streptozotocin-diabetic rats after oral or intraperitoneal administration (100).



19



20



21

Bis(maltolato)oxovanadium(IV) (**20**) and naglivan (**21**) are vanadate complexes designed to be less toxic than vanadate by increasing the absorption of vanadium from the gastrointestinal tract and thereby reducing the amount of vanadium required for a glucose lowering effect (101, 102). These compounds reduce blood glucose in streptozotocin-diabetic rats at concentrations lower than those reported to be required for vanadate or vanadyl sulfate (101-103). No long term toxicology studies have been reported for **19**- **21** compared to vanadyl sulfate.

Co-administration of vanadate with sodium 4,5 dihydroxybenzene-1,3-disulfonate, a chelating agent that mobilizes vanadium, has been proposed as a strategy for limiting the toxicity of vanadate. This disulfonate decreases the accumulation of vanadium in various tissues without diminishing the anti-diabetic effects of vanadate, suggesting that this may be an effective strategy for reducing toxicity (104,105).

The mechanism of glucose lowering by vanadate is an area of current investigation. Vanadate is a phosphotyrosine phosphatase inhibitor capable of inhibiting the dephosphorylation of the insulin receptor. However, the insulin mimetic action and glucose lowering in ob/ob or db/db mice do not appear to depend on altering the phosphorylation state of either the insulin receptor or IRS-1 (94,106). Vanadate affects the expression of several genes involved in glucose metabolism. In streptozotocin-diabetic rats, vanadate reduces the overexpression of phosphoenol-pyruvate carboxykinase, a key regulatory enzyme in gluconeogenesis (107, 108), and increases the expression of glucokinase, L-type pyruvate kinase and phosphofructokinase (107-111). Vanadate also increases the levels of fructose 2,6-bisphosphate, a key regulator of glycolysis, and normalizes the ratio of 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase, the enzyme pair that regulates the levels of fructose 2,6-bisphosphate (109, 111, 112). These changes in enzyme expression are consistent with the insulin

mimetic and glucose lowering activities of vanadate, but it is not clear that they represent the molecular mechanism of vanadate action.

Amylin - Amylin is being investigated for the treatment of both IDDM and NIDDM. Phase II trials are in progress in patients with IDDM to determine whether amylin replacement reduces the risk of hypoglycemia associated with insulin therapy (113). AC-137, which is human amylin with proline substitutions at positions 25, 28 and 29 to limit aggregation, is being used for these trials. AC-137 has been administered to fasted IDDM subjects as a continuous i.v. infusion for 2 hours at 15, 50 and 150 μg/hr, or as an i.v. bolus of 30, 100 and 300 μg (113). Thirty minutes after administration of AC-137, a meal was given and plasma glucose was measured over a 4 hour period. There was no effect of AC-137 on baseline glucose levels, which contrasts with the elevation that might have been expected based on amylin's mechanism of action. AC-137 decreased postprandial glucose levels significantly in the 50 and 150 μg/hr groups, but not in the 15 μg/hr group. The glucose lowering effects of AC-137 are attributed to delayed gastric emptying. Inhibition of gastric acid secretion (114) and gastric emptying (115, 116) have also been observed in dogs and rats. It is unclear whether these effects are mediated by the amylin receptor or the CGRP-I receptor, since both acid secretion and emptying are sensitive to the CGRP antagonist, CGRP(8-37) (117,118).

Amylin antagonism is of interest in terms of treatment of NIDDM based on the inhibition of insulin action in muscle and liver by amylin (119). AC-187 is a peptidic amylin antagonist composed of the amino acid sequence of salmon calcitonin (8-32) and the C-terminus of amylin. AC-187 suppresses the rise in plasma lactate in response to glucose or amylin infusion into rats, suggesting inhibition of amylin action in muscle (120).

In addition to inhibiting insulin action in muscle and liver, amylin inhibits insulin secretion by a paracrine mechanism (21-123). Amylin antagonists, such as amylin (8-37) and salmon calcitonin (8-32), enhance glucose- or arginine-stimulated insulin secretion (120, 124, 125). Infusion of amylin (8-37) into anesthetized rats lowers blood glucose and increases insulin secretion (124). Thus, amylin antagonists may lower plasma glucose in NIDDM by effects on both insulin action and insulin secretion. Two injectable, peptidic amylin antagonists are in early clinical trials (126).

Conclusions - This review has focused on areas of intervention that are currently being explored in humans. Data should emerge within the next few years, which will provide insight into the effectiveness of these treatments. Future directions for liver-based approaches could focus on reducing glucose output from this tissue. In the pancreas, new opportunities for enhancing insulin secretion may result from research on glucose signaling and the steps involved in the exocytosis of insulin. In addition, immunotherapy may delay the onset of IDDM by preventing the autoimmune destruction of the pancreas. Understanding of the mechanism of information flow from the insulin receptor to translocation of glucose transporters in peripheral tissues is expanding rapidly. These avenues of research lay the groundwork for future drug discovery approaches directed toward the treatment of diabetes.

References

1. R.J. Rubin, W.M. Altman, and D.N. Mendelson, J.Clin.Endocrinol.Metab., 78, 809A (1994).
2. Diabetes Control and Complications Trial Research Group, New Engl.J.Med., 329, 977 (1993).
3. R.A. DeFronzo, Diabetes, 37, 667 (1989).
4. J.M. Olefsky, W.T. Garvey, R.R. Henry, S. Matthaei and G.R.Freidenberg, Am.J.Med., 85 (Suppl. 5A), 86 (1988).
5. C.R. Kahn, Metabolism, 27 (Suppl. 2), 1893 (1978).
6. L.C. Groop, Diabetes Care, 15, 737 (1992).
7. J.R. White, Am.Pharm., NS32, 39 (1992).
8. W.J. Malaisse and P. Lebrun, Diabetes Care, 13 (Suppl. 3), 9 (1990).

9. J.C. Henquin in "New Antidiabetic Drugs", C.J. Bailey and P.R. Flatt, Eds.; Smith-Gordon, London, 1990, p. 93.
10. H.G. Coore and P.J. Randle, Biochem.J., 93, 66 (1964).
11. R.E. Miller, Endocrine Rev. 2, 471 (1981).
12. S.L.F. Chan, Clin. Science, 85, 671 (1993).
13. N.G. Morgan, Exp.Opin.Invest.Drugs, 3, 561 (1994).
14. I. Angel, A. Grosset, G. Perrault, H. Schoemaker and S.Z. Langer, Eur.J.Pharmacol., 234, 137 (1993).
15. J.-C. Jonas, T.D. Plant, I. Angel, S.Z. Langer and J.-C. Henquin, Eur.J.Pharmacol., 54, 27 (1994).
16. J.-C. Jonas, M.-J. Garcia-Barrado, I. Angel and J.-C. Henquin, Eur. J.Pharmacol., 264, 81 (1994).
17. I. Berlin, S. Chalon, P. Rosenzweig, E. Fuseau and A.J. Puech, Fundam.Clin.Pharmacol., 7, 348 (1993).
18. I. Berlin, P. Rosenzweig, S. Chalon, E. Fuseau, C. Landult, F. Cesselin, C. Blacker and A.J. Puech, Clin.Pharmacol.Ther., 55, 338 (1994).
19. M.J. Berge, J.L. Beeley and A.M. Cawthorne, WO 9309113 (1992).
20. W. Draber and H. Bischoff, EP 552,631 (1993).
21. A. Kashiwagi, Y. Harano, M. Suzuki, H. Kojima, M. Harada, Y. Nishio and Y. Shigeta, Diabetes, 35, 1085 (1986).
22. S. Kawazu, K. Suzuki and J. Negishi, J. Ishii, H. Sando, H. Katagiri, Y. Kanazawa, S. Yamanouchi, Y. Akanuma, H. Kajinuma, K. Suzuki, K. Watanabe, T. Itoh, T. Kobayashi and K. Kosaka, Diabetes, 36, 221 (1987).
23. A.D. Struthers, D.C. Brown, M.J. Brown, B. Schumer and S.R. Bloom, Clin.Endocrinol., 23, 539 (1985).
24. C.G. Ostenson, J. Pigon, J.C. Doxey and S. Efendic, J. Clin.Endocrinol.Metab., 67, 1054 (1988).
25. L.C. Meurer, R.L. Tolman, E.W. Chapin, R. Sperstein, P.P. Vicrio, M.M. Zrada and M. MacCoss, J.Med.Chem., 35, 3845 (1992).
26. Y. Kunoh, A. Iguchi, M. Gotoh, T. Nomura, M. Shibata and N. Sakamoto, Arch. Int. Pharmacodyn., 280, 302 (1986).
27. Y. Kunoh, H. Ogawa, M. Nagasaka, H. Asai, A. Iguchi, A. Niki and N. Sakamoto, Arch.Int.Pharmacodyn., 298, 276 (1989).
28. H.J. Breslin, M.J. Kukla, R.W. Tuman, M.C. Rebarchak and C.R. Bowden, J.Med.Chem., 36, 1597 (1993).
29. R.W. Tuman, G.F. Tutwiler and C.R. Bowden in "New Antidiabetic Drugs", C.J. Bailey and P.R. Flatt, Eds.; Smith-Gordon, London, 1990, p. 163.
30. B. Thorens and G. Waeber, Diabetes, 42, 1219 (1993).
31. C. Orskov, M. Bersani, A. H. Johnsen, P. Hojrup and J.J. Holst, J.Biol.Chem. 264, 12826 (1989).
32. S. Mojsov, M.G. Kopczynski and J.F. Habener, J.Biol.Chem., 265, 8001 (1990).
33. K. Adelhorst, B.B. Hedegaard, L.B. Knudsen and O. Kirk, J.Biol.Chem., 269, 6275 (1994).
34. S.A. Hjorth, K. Adelhorst, B.B. Pedersen, O. Kirk and T.W. Schwartz, J.Biol.Chem., 269, 30121 (1994).
35. Y. Watanabe, K. Kawai, S. Ohashi, C. Yokota, S. Suzuki and K. Yamashita, J.Endocrin., 140, 45 (1994).
36. C. Orskov, A. Wettergren and J.J. Holst, Diabetes, 42, 658 (1993).
37. M. Nauck, F. Stockman, R. Ebert and W. Creutzfeldt, Diabetologia, 29, 46 (1986).
38. M. Hirota, M. Hashimoto, M. Hiratsuka, C. Ohboshi, S. Yoshimoto, M. Yano, A. Mizuno and K. Shima, Diabetes Res.Clin.Prac., 9, 179 (1990).
39. C. Orskov, J. Jeppesen, S. Madsbad and J.J. Holst, J.Clin.Invest., 87, 415 (1991).
40. M.A. Nauck, M.M. Heimesaat, C. Orskov, J.J. Holst, R. Ebert and W. Creutzfeldt, J.Clin.Invest., 91, 301 (1993).
41. D.M. Nathan, E. Schreiber, H. Fogel, S. Mojsov and J.F. Habener, Diabetes Care, 15, 270 (1992).
42. M. Gutniak, C. Orskov, J.J. Holst, B. Ahren and S. Efendic, N.Engl.J.Med., 326, 1316(1992).
43. D.A. D'Alessio, S.E. Kahn, C.R. Leusner and J.W. Ensinck, J.Clin.Invest., 93, 2263 (1994).
44. J.A. Galloway, Diabetes Care, 16 (Suppl. 3), 16 (1993).
45. D.N. Brems, L.A. Alter, M.J. Beckage, R.E. Chance, R.D. Dimarchi, L.K. Green, H.B. Long, A.H. Pekar, J.E. Shields and B.H. Frank, Protein Eng., 5, 527 (1992).
46. L. Heinemann, T. Heise, L.N. Jorgensen and A.A. Starke, Diabet.Med., 10, 535 (1993).
47. W.H. Fischer, D. Saunders, D. Brandenburg, A. Wollmer and H. Zahn, Biol.Chem. Hoppe-Seyler, 366, 521(1985).
48. H. Hartmann, E. Moesus and W. Creutzfeldt, Diabetes Res.Clin.Pract., 16, 175 (1992).
49. F. Stumpel, H. Hartmann, D. Brandenburg and W. Creutzfeldt, Diabetes Res.Clin.Pract., 9, 257 (1990).
50. D.C. Howey, R.R. Bowsher, R.L. Brunelle and J.R. Woodworth, Diabetes, 43, 396 (1994).
51. L. Vignati, J. Anderson and R. Brunelle, Diabetes, 43 (Suppl 1), 78A (1994).

52. R. Bergenstal, M. Spencer, G. Castle, C. Roethke, J. Keough, A. Lau, P. Upham and L. Deeb, Diabetes, 43 (Suppl 1), 157A (1994).
53. T. Sohda, K. Mizuno, E. Imamiya, T. Sugiyama, T. Fujita and Y. Kawamatsu, Chem.Pharm.Bull., 30, 3580 (1982).
54. A.Y. Chang, B.M. Wyse, B.J. Gilchrist, T. Peterson and A.R. Diani, Diabetes, 32, 830 (1983).
55. J.R. Colca and D.R. Morton in "New Antidiabetic Drugs", C.J. Bailey and P.R. Flatt, Eds.; Smith-Gordon, London, 1990, p. 255.
56. T. Sohda, K. Meguro, Y. Kawamatsu, H. Ikeda and T. Fujita, J.Pharm.Sci., 76, S/173 (1987).
57. Y. Momose, K. Meguro, H. Ikeda, C. Hatanaka, S. Oi and T. Sohda, Chem.Pharm.Bull. 39, 1440 (1991).
58. J.R. Colca, C.F. Dailey, B.J. Palazuk, R.M. Hillman, D. M. Dinh, G.W. Melchior and C.H. Spilman, Diabetes, 40, 1669 (1991).
59. R.W. Stevenson, N.J. Hutson, M.N. Krupp, R.A. Volkmann, G.F. Holland, M.R. Johnson, J.F. Eggler, D.A. Clark, R.K. McPherson, K. L. Hall, B.H. Danbury, E.M. Gibbs and D.K. Kreutter, Diabetes, 39, 1218 (1990).
60. D.A. Clark, S.W. Goldstein, R.A. Volkmann, J.F. Eggler, G.F. Holland, B. Hulin, R.W. Stevenson, D.K. Kreutter, E.M. Gibbs, M.N. Krupp, P. Merrigan, P.L. Kelbaugh, E.G. Andrews, D.L. Tickner, R.T. Suleske, C.H. Lamphere, F.J. Rajeckas, W.H. Kappeler, R.E. McDermott, N.J. Hutson and M.R. Johnson, J.Med.Chem., 34, 313 (1991).
61. T. Yashioka, T. Fujita, T. Kanai, Y. Aizawa, T. Kurumada, K. Hasegawa and H. Horikoshi, J.Med.Chem., 32, 421 (1989).
62. Drugs of the Future, 16, 853 (1991).
63. Drugs of the Future, 19, 890 (1994).
64. Y. Iwamoto, T. Kuzuya, A. Matsuda, T. Awata, S. Kumakura, G. Inooka and I. Shiraishi, Diabetes Care, 14, 1083 (1991).
65. S.L. Suter, J.J. Nolan, P. Wallace, B. Gumbiner and J.M. Olefsky, Diabetes Care, 15 (1992)
66. J.J. Nolan, B. Ludvik, P. Beerdsen, M. Joyce and J. Olefsky, N.Engl.J.Med., 331,1188 (1994).
67. T. Sohda, K. Mizuno, Y. Momose, H. Ikeda, T. Fujita and K. Meguro, J.Med.Chem., 35, 2617 (1992).
68. B. Hulin, D.A. Clark, S.W. Goldstein, R.E. McDermott, P.J. Dambek, W.H. Kappeler, C.H. Lamphere, D.M. Lewis and J.P. Rizzi, J.Med.Chem., 35, 1853 (1992).
69. B.C.C. Cantello, M.A. Cawthorne, D. Haigh and R.M. Hindley, Bio.Med.Chem. Let., 4, 1181 (1994).
70. B.C.C. Cantello, M.A. Cawthrone, G.P. Cottam, P.T. Duff, D. Haigh, R.M. Hindley, C.A. Lister, S.A. Smith and P.L. Thurlby, J.Med.Chem., 37, 3977 (1994).
71. G. Regnier, Y. Chatenay, J. Duhault and J. Espinal, EP 593,348 (1994).
72. S. Yano, K. Ogawa and M. Fukushima, WO 9422857 (1994).
73. G. de Nanteuil, J. Duhault, J. Espinal and D. Ravel, EP 559,571 (1993).
74. G. de Nanteuil, J. Duhault, D. Ravel and Y. Herve, EP 528,734 (1993).
75. T. Sohda, H. Ikeda, S. Imai and Y. Momose, EP 605,228 (1994).
76. T. Yoshioka, S. Yoshioka, Y. Fujiwara and H. Horikoshi, EP 590,793 (1994).
77. J.W. Ellingboe, T.R. Alessi, T.M. Dolak, T.T. Nguyen, J.D. Tomer, F. Guzzo, J.F. Bagli and M.L. McCaleb, J.Med.Chem., 35, 1176 (1992).
78. K. Niigata, T. Takahashi, T. Maruyama, T. Suzuki, K. Maeno, K. Onda, T. Kontani, O. Noshiro, R. Koike, A. Shimaya, J. Irie and S. Nagai, WO 9425448 (1994).
79. R.M. Hindley, WO 920252 (1992).
80. G.D. Williams, A. Deldar, W.H. Jordan, C. Gries, G. Long and R.D. Dimarchi, Diabetes, 52 (Suppl. 1), 59A (1993).
81. K. Megura and T. Fujita, EP 193,256 (1986).
82. J. Duhault, F. Lacour, M. Boulanger, O. Della-Zuana, D. Ravel, M. Wierzbicki and J. Espinal, Diabetologia, 37, 969 (1994).
83. C.J. Bailey, Diabetes Care 15, 755 (1992).
84. B.C. Pressman, J. Biol. Chem., 238, 401 (1963).
85. G. Schafer in "Inhibitors of Mitochondrial Function" M. Erecinska and D.F. Wilson, Ed., Pergamon Press, New York, N.Y., 1981, p. 165.
86. M.D. Meglasson, J.M. Wilson, J.H. Yu, D.D. Robinson, B.M. Wyse and C.J. deSouza, J. Pharmacol.Exp.Ther., 266, 1454 (1993).
87. Y. Shechter and S.J.D. Karlish, Nature, 284, 556 (1980).
88. H. Sakurai, S. Shimomura, K. Fukuzawa and K. Ishizu, Biochem.Biophys.Res.Commun., 96, 293 (1980).
89. C.E. Heyliger, A.G. Tahiliani and J.H. McNeill, Science, 227, 1474 (1985).
90. J. Meyerovitch, Z. Farfel, J. Sack and Y. Shechter, J.Biol.Chem., 262, 6658 (1987).
91. S. Ramanadham, J.J. Mongold, R.W. Brownsey, G.H. Cros and J.H. McNeill, Am.J.Physiol., 257, H904 (1989).
92. S. Dai, K.H. Thompson and J.H. McNeill, Pharmacol.Toxicol., 74, 101 (1994).
93. S.M. Brichard, C.J. Bailey and J.-C. Henquin, Diabetes, 39, 1326 (1990).
94. J. Meyerovitch, P. Rothenberg, Y. Shechter, S. Bonner-Weir and C.R. Kahn, J.Clin.Invest., 87, 1286 (1991).

95. L. Rossetti, N. Cohen, M. Halberstam, P. Shlimovich, M. Hu and H. Shamoon, Can.J.Physiol.Pharm., 72 (S3), 11 (1994).
96. A.B. Goldfine, F. Folli Patti, M.E., D.C. Simonson and C.R. Kahn, Can.J.Physiol.Pharm., 72 (S3), 11 (1994).
97. U.H. Malabu, S. Dryden, H.D. McCarthy, A. Kilpatrick and G. Williams, Diabetes, 43, 9 (1994).
98. J.M. Llobet and J.L. Domingo, Toxicol.Lett., 23, 227 (1984).
99. S. Dai, K.H. Thompson, E. Vera and J.H. McNeill, Pharmacol.Toxicol., 75, 265(1994).
100. H. Watanabe, N. Masami, K. Komazawa and H. Sakurai, J.Med.Chem., 37, 876 (1994).
101. J.H. McNeill, V.G. Yuen, H.R. Hoveyda and C. Orvig, J.Med.Chem., 35, 1489 (1992).
102. M.C. Cam, G.H. Cros, J.-J. Serrano, R. Lazaro and J.H. McNeill, Diabetes Res.Clin.Pract ., 20, 111 (1993).
103. V.G. Yuen, C. Orvig and J.H. McNeill, Can.J.Physiol.Pharmacol., 71, 263 (1993).
104. J.L. Domingo, M. Gomez, D.J. Sanchez, J.M. Llobet and C.L. Keen, Life Sci., 50, 1311(1992).
105. J.L. Domingo, D.J. Sanchez, M. Gomez, J.M. Llobet and J. Corbella, Vet.Hum.Toxicol., 35, 495 (1993).
106. H.V. Strout, P.P. Vicario, R. Saperstein and E.E. Salter, Endocrinology, 124, 1918 (1989).
107. S.M. Brichard and D.B.J. Girard, Mol. Cell. Endocrinology, 91, 91 (1993).
108. A. Valera, J.E. Rodriquez-Gil and F. Bosch, J.Clin.Invest., 92, 4 (1993).
109. M. Miralpeix, E. Carballo, R. Bartrons, K. Crepin, L. Hue and G.G. Rousseau, Diabetologia, 35, 243 (1992).
110. A.K. Saxena, P. Srivastave and N.Z. Baquer, Eur.J.Pharmacol., 216, 123 (1992).
111. M. Sochor, S. Kunjara, M. Ali and P. McLean, Biochem.Int., 28, 525 (1992).
112. M. Saad, E. Araki, M. Miralpeix, P.L. Rothenberg, M.F. White and C.R. Kahn, J.Clin.Invest., 90, 1839 (1992).
113. O. Kolterman, A. Glottlieb and C. Moyses, Diabetes, 43, 78A (1994).
114. F. Guidobono, M. Coluzzi, F. Pagani, A. Pecile and C. Netti, Peptides, 15, 699 (1994).
115. A. Young, P. Carlo, P. Smith, W. Vine and B. Gedulin, Diabetes, 43, 174A (1994).
116. K. Brown, A. Menius, E. Sandefer, J. Edwards and M. James, Diabetes, 43, 172A (1994).
117. D.C. Lawson, C.R. Mantyh and T.N. Pappas, Digest.Dis.Sci., 39, 1405 (1994).
118. V. Plourde, S. Stpierre, A. Fournier and Y. Tache, Life Sciences, 52, 857 (1993).
119. A.A. Young, J. Cellular Biochem., 55S, 12-18 (1994).
120. A.A. Young, B. Gedulin, L. Gaeta, K.S. Prickett, K. Beaumont, E. Larson and T.J. Rink, FEBS Lett, 343, 237 (1994).
121. R.A. Silvestre, E. Peiro, P. Degano, P. Miralles and J. Marco, Regul.Pept., 31, 23 (1990).
122. P. Degano, R.A. Silvestre, M. Salas, E. Peiro and J. Marco, Regul.Pept., 43, 91 (1993).
123. R.A. Silvestre, M. Salas, O. Garciahermida, T. Fontela, P. Degano and J. Marco, Regul.Pept., 50, 193 (1994).
124. W.M. Bennet, C.S. Beis, M.A. Ghatei, P. Byfield and S.R. Bloom, Diabetologia, 37, 436 (1994).
125. Z.-L. Wang, W.M. Bennet, M.A. Ghatei, P.G.H. Byfield, D.M. Smith and S.R. Bloom, Diabetes, 42, 330 (1993).
126. J. Shrine, BioWorld Today, 6, 1 (1995).

Chapter 18. Gonadotropin Releasing Hormone Antagonists

Mark T. Goulet
Merck Research Laboratories, Rahway, NJ 07065

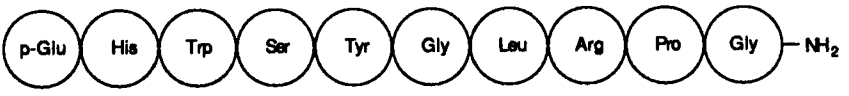
Introduction - Since its discovery in 1971, gonadotropin releasing hormone (GnRH, LH-RH, 1) and its synthetic analogs have been administered for an increasing variety of clinical indications (1,2). Neglecting for the purpose of this chapter the role of GnRH as a therapeutic, both GnRH agonists and antagonists have found utility in treatments where a reversible suppression of the pituitary-gonadal axis is desired. Sometimes referred to as 'biochemical castration', GnRH-based therapies have proven effective in the treatment of endometriosis, uterine fibroids, polycystic ovarian disease, precocious puberty, and several gonadal steroid-dependent neoplasia, most notably cancers of the prostate, breast, and ovary. GnRH agonists and antagonists have also been utilized in various assisted fertilization techniques and have been investigated as a potential contraceptive in both men and women. The therapeutic potential of GnRH analogs has been the subject of several recent reviews (3-9).

Although they exert discrete biochemical effects, both GnRH agonists and antagonists act to lower the circulating levels of follicle-stimulating hormone (FSH), leutenizing hormone (LH) and, as a consequence of the latter, the gonadal hormones testosterone and estradiol. Prolonged stimulation of the GnRH receptor with GnRH agonists results in receptor down-regulation and eventual desensitization of the pituitary, whereas GnRH antagonists act by non-productive receptor occupation. GnRH agonists typically require 10-14 days to elicit their intended pharmacological outcome. During this time a transient rise in gonadal hormone production, or 'flare effect', occurs followed by reduction of these hormones to therapeutic levels. GnRH antagonists have the advantage of immediate pituitary response without the sometimes symptom-exacerbating hormone flare. Several reviews have appeared that chronicle advances made in the GnRH analog field (10-12). This chapter will survey recent progress in the area of GnRH antagonist design.

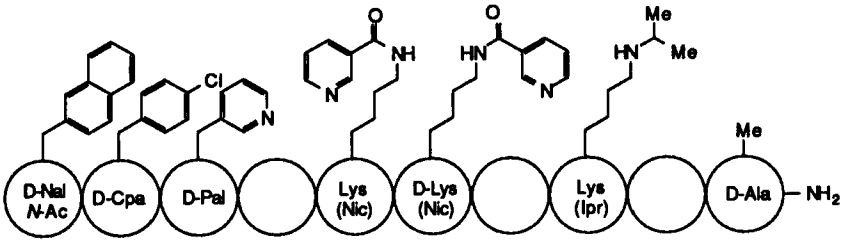
Decapeptide GnRH Antagonists - Like the GnRH agonists, most antagonists are decapeptide analogs of the natural hormone. However, unlike GnRH agonists, extensive modifications of up to seven amino acids are often required to achieve the desired properties in an antagonist. Historically, the development of GnRH antagonists has been characterized by three distinct generations based on the ability of these compounds to 1) demonstrate *in vivo* efficacy, 2) eliminate the histamine-releasing activity associated with these structures and 3) improve the safety profile and duration of action (13,14). Several "third generation" antagonists including: antide (Nal-Lys, 2); cetrorelix (SB-75, 3); ganirelix (RS26306, 4); detirelix (RS68439, 5); and A75998 (6) are undergoing clinical trials, however, no GnRH antagonists have been approved as a therapeutic (15-19). Post "third generation" issues being addressed include aqueous solubility, increased duration of action, and oral activity. The general approach has been to modify structures such as 2 with a concentration on position 1 and positions 5-8.

A study of the effect of bicyclic aromatic groups attached by acylation to small, non-aromatic amino acids at position 1 was conducted to find potential replacements for *N*-Ac-D-Nal[1] present in most antagonists (20). 2-Naphthoic acid derivatives of position 1 were found to be substantially less active than *N*-Ac-D-Nal[1], however, incorporation of a nitrogen into the aromatic group as in the 3-quinoline- and

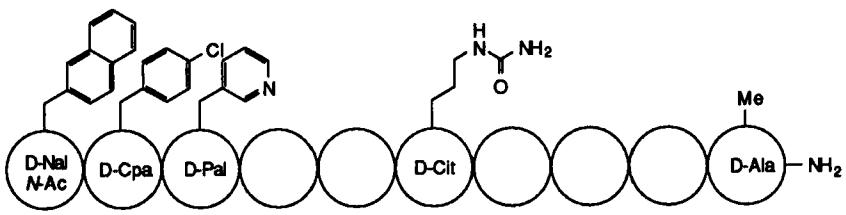
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.



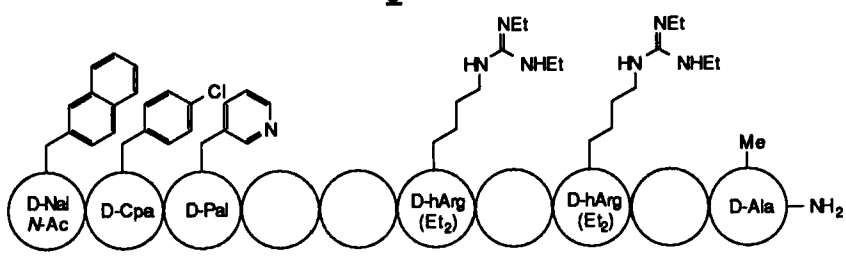
1



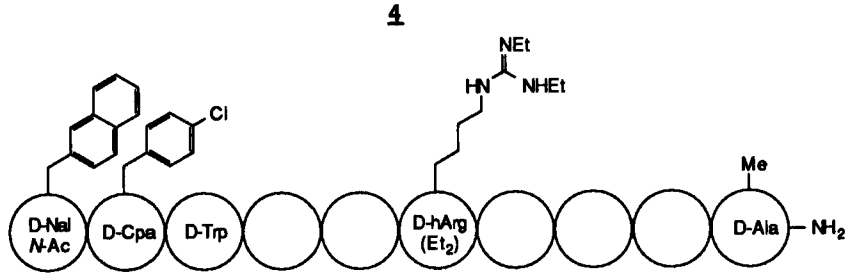
2



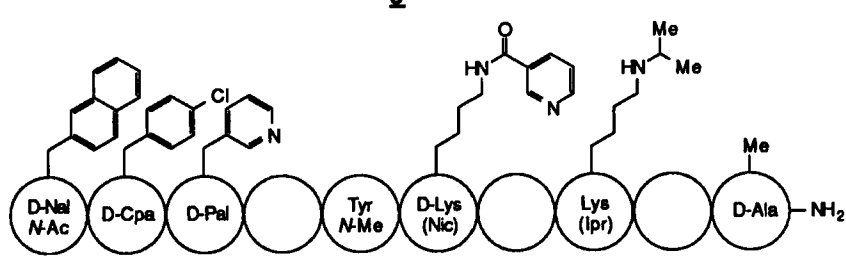
3



4

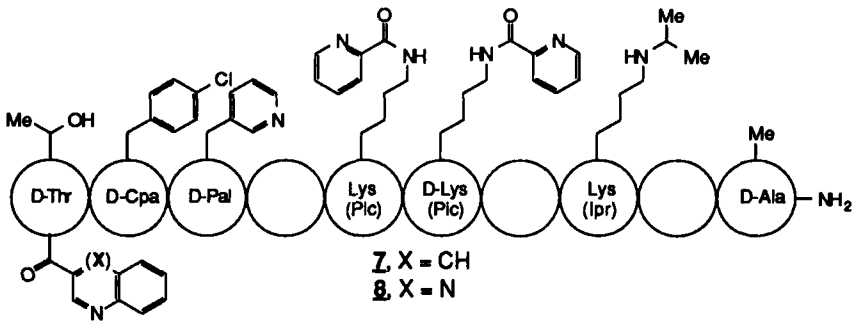


5



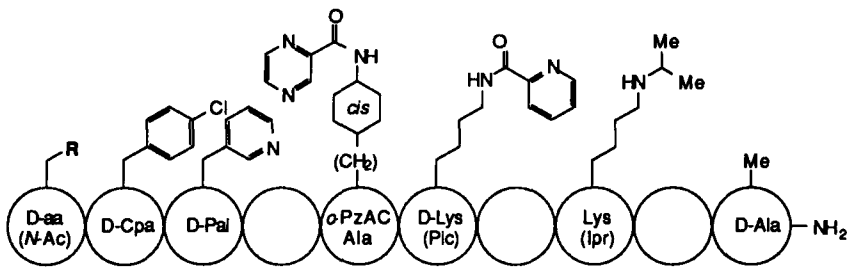
open circles = identical to GnRH 6

2-quinoxalinecarboxamide modified DThr[1], **7** and **8** respectively, gave adequate antagonist activity in a rat antiovulatory assay (AOA ED_{50} = 2.5 μg/rat) with improved histamine release and solubility profiles compared to the *N*-Ac-D-Nal[1] parent.

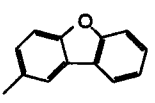


7, X = CH
8, X = N

Replacement of *N*-Ac-D-Nal[1] with *N*-Ac-D-*O*-phenyltyrosine as in **9** reduced the AOA potency relative to parent (38% vs 90% @ 0.5 μg/rat) (21). In contrast, substitution with a more rigid aromatic sidechain such as *N*-Ac-D-3-(2-dibenzofuranyl)alanine (**10**) was found to retain the AOA activity of parent.



9, R =

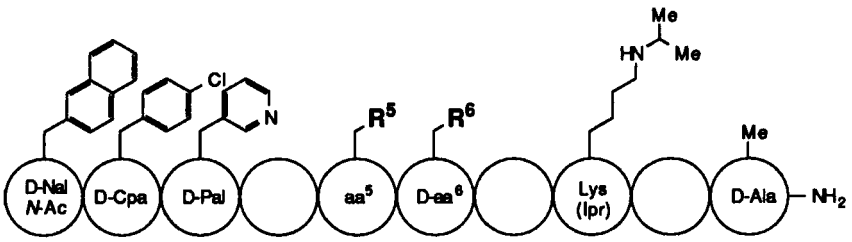


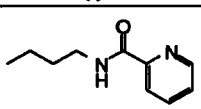
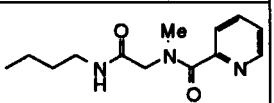
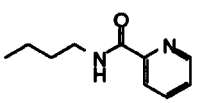
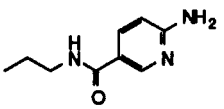
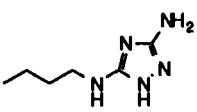
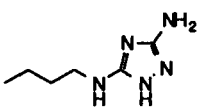
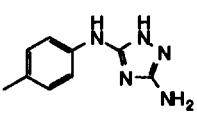
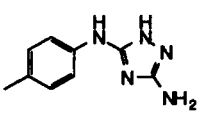
10, R =

N-Methyl substitution of the GnRH agonists leuprolide (22), deslorelin (23), and nafarelin (24) has been conducted to determine the effect of this modification on GnRH receptor binding, LH release, and stability toward enzymatic and intestinal degradation (25). In general, this modification was found to reduce potency in both the receptor binding and LH release assays, with certain position 2, 3, and 8 *N*-methyl analogs exhibiting antagonist activity. *N*-Methylation of leuprolide at position 2,3,4, or 5 markedly enhances the stability toward degradation by chymotrypsin, whereas, this modification in residues 6-10 had no effect. Similarly, stability toward enzymatic degradation in a rat jejunum sac model was enhanced in several analogs containing an *N*-methyl residue at positions 2-6, with *N*-MeSer[4] leuprolide exhibiting a half-life 20-fold greater than parent. Low clearance values (rat) were also observed for the *N*-MeTyr[5] analogs suggesting that this modification can also reduce the rate of metabolism.

Substitution of Lys(Nic)[5] in **2** with *N*-MeTyr[5] gave analog **6** which exhibits increased receptor binding and *in vitro* LH-release inhibition (19). This modification also improves the aqueous solubility compared to **2** (20 vs 1 mg/mL), however, a 26-fold increase in histamine releasing potency is also observed.

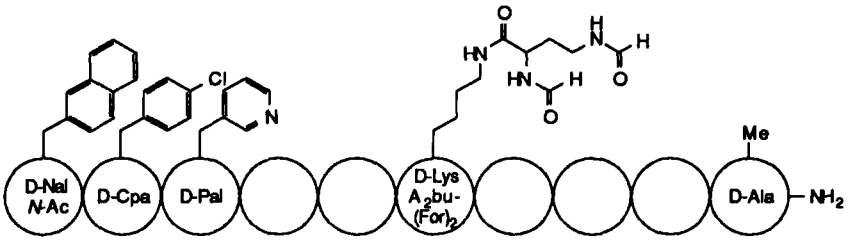
The N^{ω}-acylated amino acids at positions 5 and 6 of **2** have been modified in an attempt to improve aqueous solubility and alleviate problems associated with formulation. While most acyl variants of position 5 alone were found to reduce AOA potency, analogs containing D-Lys (PicSar) at position 6 with either $Lys(Pic)^5$ (sartide, **11**) or Tyr^5 (not shown) maintained the antagonist activity of parent (26,27). The aqueous solubility of these analogs is significantly greater than **2** (50-100 vs 1 mg/mL) but with an accompanying 10-fold increase in histamine release. Addition of a primary amine in the N^{ω}-acyl group, as in nictide (**12**), increases solubility (>8 mg/mL) with only a modest effect on histamine activity (28). In another study it was found that incorporation of 3-amino-1H-1,2,4-triazolyl (atz) modified amino acids at both positions 5 and 6 could provide analogs of **2** with improved properties (29). Azalines A (**13**) and B (**14**) containing (atz) derivatives of Lys and 4-amino-Phe, respectively, are quite potent antagonists (AOA) with low histamine releasing activity and up to 20-fold greater aqueous solubility compared to **2**. When dosed subcutaneously at 10 or 50 µg/rat, analog **14** exhibited a duration of action for LH suppression in the castrated rat similar to **2**. However, upon i.v. administration (250 µg) **14** was active for >60h, whereas **2** lost effectiveness within 24h. In similar experiments, **14** has demonstrated superior duration to both **3** and **4**.



No.	Position 5	R^5	Position 6	R^6
11	Lys(Pic)		DLys(PicSar)	
12	Lys(Pic)		DOrn(6ANic)	
13	Lys(atz)		DLys(atz)	
14	Aph(atz)		DAph(atz)	

Analogs of **3** where the $D\text{-}Cit^6$ has been replaced by various N^{ω}-acylated D-Lys residues have been described (30). Several members of this class, such as T-159 (**15**), are potent GnRH antagonists (AOA ED_{100} = 0.75 µg/rat) with >24h duration of

action for the reduction of LH (2.5 μg/rat) and FSH (5 μg/rat) upon sc administration to castrated rats. In intact rats, **15** was able to reduce the serum sex hormone concentration to castration levels for >48h at a dose of 100 μg/rat. Structures of this type were also found to be similar to **3** in their ability to induce a cutaneous anaphylactoid reaction as an *in vivo* model of histamine release.

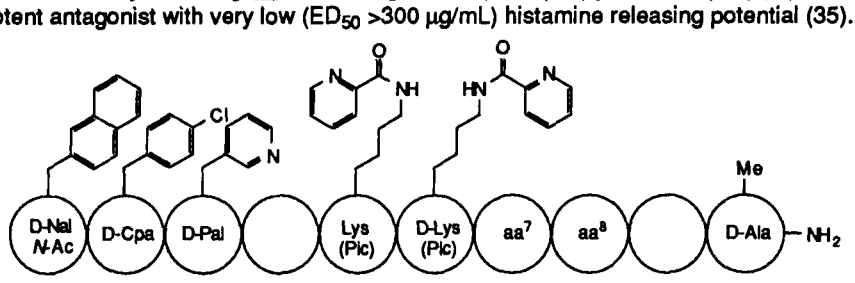


15

Antarelix (EP 24332) is another analog of **3** containing a D-hCit^6 and D-Lys(Ipr)^8 that is reported to be entering development (31). These modifications impart improved aqueous solubility (10 mg/mL) while maintaining modest *in vitro* histamine releasing potency.

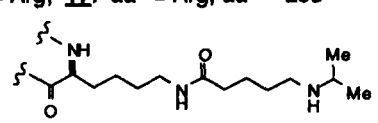
A systematic study of position 6 and Arg^8-modified antagonists has been conducted in an attempt to reduce the histamine releasing potential of the potent Arg^6-containing class of antagonists (32,33). It was found that a *N*, *N'*-dialkylhomoarginine residue at both positions 6 and 8, as in the clinical candidate **4**, increased the potency while maintaining acceptable histamine release levels. Substitution of hArg$(CH_2CF_3)_2^8$ for hArg$(Et)_2^8$ decreased substantially the ability of these analogs to release histamine (*in vitro* EC_{50} >1000 μg/mL) but with accompanying attenuation of AOA activity.

Translocation of the basic amino acid residue from position 8 to position 7 can reduce the histamine releasing activity in an antagonist design (34). This modification, however, is often accompanied by substantial loss of antagonist potency. An interesting exception is antagonist **16** where a Leu^7-Arg^8 → Arg^7-Leu^8 exchange (**17**) resulted in only a 2.5-fold loss of AOA potency and a 10-fold decrease in histamine release activity. Analog **18** containing a N^{ω}-(6-isopropylaminocaproyl)Lys^8 is a potent antagonist with very low (ED_{50} >300 μg/mL) histamine releasing potential (35).



16: aa^7 = Leu, aa^8 = Arg; **17**: aa^7 = Arg, aa^8 = Leu

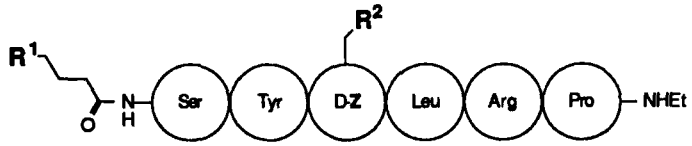
18 aa^7 = Leu, aa^8 =



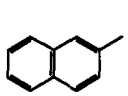
Toward the *de novo* design of improved antagonist structures, a computational study of likely drug conformations has been conducted. Energy calculations using the ECEPP force field were performed on GnRH and several agonist and antagonist structures (36,37). GnRH agonists are predicted to have a conformation very close to that of the native hormone where it is proposed that a "surface" in the shape of a polygon defined by the hydrophobic sidechains of positions 1, 3, 5, 7 and 9 surrounding the His^2 side chain constitutes the bioactive conformation. In this model, antagonists can maintain a similar overlap with GnRH in the *N*-terminal tripeptide region but are thought to differ substantially in the "turn" positions 5-8.

In an experiment designed to compare the interaction of agonists (Ag) and antagonists (Antag) with the GnRH receptor, rat pituitary membranes were incubated with either a GnRH Ag or Antag containing a ^{125}I-photoaffinity label at position 6 (38). Trypsin digestion of the GnRH•Ag and GnRH•Antag photoadducts revealed different fragment patterns indicating that these ligands bind the receptor in different orientations. From this finding, the authors caution that separate strategies should be employed for the design of agonists and antagonists.

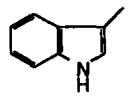
Reduced-Size Peptide Antagonists - Hexapeptide analogs of GnRH corresponding to positions 4-9 have been investigated (39). It was found that subtle perturbations of the sequence: **X**-(CONH)-Ser-Tyr-(D)-**Z**-Leu-Arg-Pro-NHEt could have profound effects on receptor binding and biological response (Ag vs Antag). For example the most potent antagonist discovered in this study (**19**, pA_2 = 9.25) differed structurally from the best agonist (**20**, p*D2* = 7.35) by a naphthalene → indole replacement on the two variable sidechains. Also, a change in the alkyl tethering group for R^1 could cause the biological response to switch in several structural series. Hexapeptide antagonist **19** has a receptor binding affinity 4-fold greater than GnRH, and *in vivo* activity by i.v. infusion has been demonstrated for both **19** and **20**.



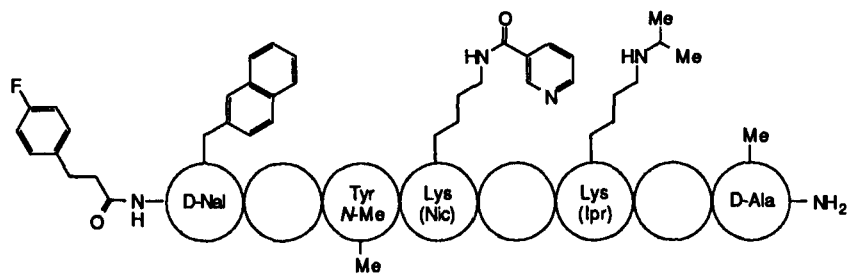
19: R^1, R^2 =



20: R^1, R^2 =

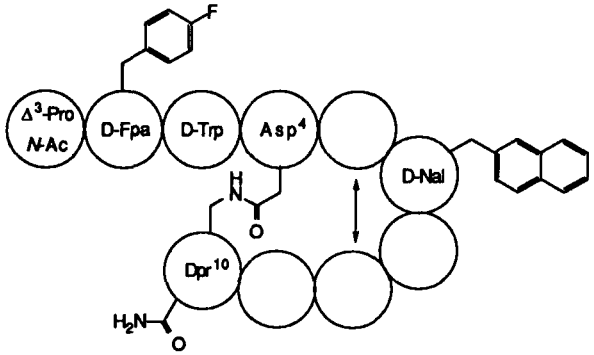


An octapeptide antagonist (A76154, **21**) corresponding to the C-terminus of GnRH has been developed that lowers LH in the castrated rat with equal efficacy to the decapeptide structure **6** and with similar histamine release activity (40). Analog **21** shows good *in vivo* activity upon sc bolus administration indicating that a greater duration of action is achieved than with the hexapeptide **19**.



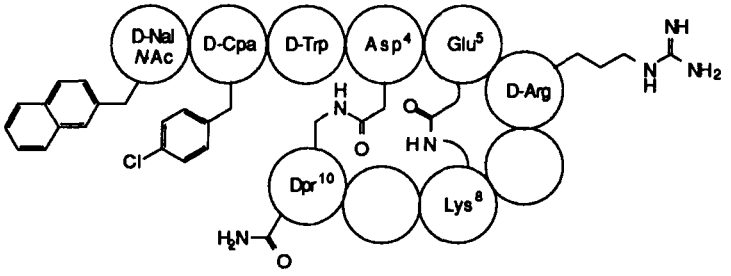
21

Conformationally-Constrained Peptides - Although GnRH is thought to be essentially unstructured in solution, cyclic analogs have been investigated that might mimic the type II' β-turn present in the presumed bioactive conformation (41,42). Cyclo(4-10) structures such as **22** exhibit antagonist activities approaching those of the acyclic parent (80% AOA @ 10 μg/rat; 43,44). Analysis of **22** by NMR and computational methods predicts the existence of a type II' β-turn around positions 6 and 7 with transannular contacts between positions 5 and 8 (45,46). This information was used in



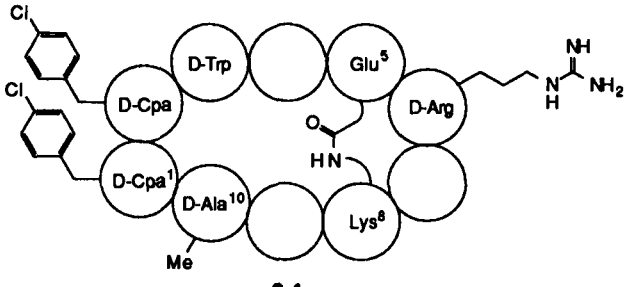
22

the design of bicyclic antagonists containing a covalent bridge between residues 5 and 8 (44). Structural analysis of this analog revealed a conformation similar to **22** and suggested favorable interactions between the *N*-terminal residues and the 5-8 bridge (47,48). Bicyclic analog **23** was also found to be 2-fold more potent than **22** *in vivo* (80% AOA @ 5 μg/rat).



23

A structural analog of **23**, containing an identical sequence in positions 5-9 but with the N- and C-termini joined in a second ring, was predicted to have an entirely distinct global conformation (49). Energy calculations performed on analog **24** predicted a



24

β- sheet structure in the region of residues 6-8 and 1-3 with turns occurring at positions 4-5 and 9-10. Analog **24** was found to be a much weaker antagonist (50% AOA @ 200μg/rat) than **23**.

GnRH Antagonists in Humans Recent reports on the administration of GnRH antagonists to humans have concentrated primarily on the issues of efficacy, as measured by immunoreactive gonadotropin and sex hormone levels, and duration of action. For example, the ability of **2** to suppress LH, FSH and testosterone in healthy men at single doses up to 50 μg/kg, sc was found to be dose dependent and similar to that of the well-studied antagonist Nal-Glu (50). In other studies, antagonist **3** was found to decrease LH and testosterone in men by 66% and 72%, respectively, at a dose of 40μg/kg (51,52). From these data it would appear that **3** is more potent than **2** with a similar (24h) duration of action, however, a head-to-head comparison of these drugs in humans has not been conducted (53-55). In postmenopausal women 300 μg of **3** was similarly effective at lowering serum LH levels when administered sc, i.m. or i.v. (56). Substantially reduced LH (90%) and FSH (70%) levels could be maintained over a 3 day period without any observable side-effects by administration of 300 μg, sc every 12 hours. A seven-day study in normal cycling women dosed daily (sc) with 3 mg of **3** resulted in a similar reduction of the gonadotropins with serum estradiol depleted to 18% of the pretreatment level (57). A comparison of antagonists **4** and **5** in postmenopausal women has shown **4** to be approximately 5-fold more potent with a 73% and 38% reduction in LH and FSH, respectively, achieved at a single dose of 3 mg, sc (17,58,59). In this study, the serum level of gonadotropins were significantly reduced for more than 72 hours.

In preliminary investigations, antagonist **3** has proven to be an effective agent for use in certain assisted reproduction protocols (60,61). Its use in the treatment of prostate cancer in man, as well as, in animal models of human breast and epithelial ovarian cancers have also produced encouraging results for the utility of "third generation" GnRH antagonists in treatment of these sex-hormone related diseases (62-65). In recognition of the expanding application of GnRH analog-based therapies, the Food and Drug Administration has recently issued guidelines for the pre-clinical testing of these agents (66).

Conclusions Since 1971, more than 3000 analogs of GnRH have been prepared in the search for receptor agonist and antagonist drugs related to the structure of this important neuropeptide hormone. In the recent past, this effort has concentrated on GnRH antagonists with an emphasis on improving selectivity and duration of action. Several antagonists are currently under clinical evaluation; however, while results thus far look promising it remains unclear whether an optimum balance of potency, toxicity, duration and convenient formulation has been attained in these drugs. Individually, these properties have been improved in many of the post-third generation antagonists described in this review, yet further testing will be required to determine if the newer designs have gained any overall improvement.

References

1. H. Matsuo, Y. Baba, R.M. Nair, A. Arimura and A.V. Schally, Biochem. Biophys. Res. Commun., 43, 1334 (1971).
2. K. Amoss, R. Burgus, R. Blackwell, W. Vale, R. Fellows and R. Guillemin, Biochem. Biophys. Res. Commun., 44, 205 (1971).
3. A.S. Dutta, Drugs Future, 13, 43 (1988).
4. M. Filicori and C. Flamingni, Drugs, 35, 63 (1988).
5. A.J. Friedman in "Gonadotropin Releasing Hormone Analogs Applications in Gynecology;" R.L. Barbieri and A.J. Friedmann, Eds; Elsevier, New York, N.Y., 1990, p. 1.
6. P.M. Conn and W.F. Crowley, Jr., N. Engl. J. Med., 324, 93 (1991).
7. R.L. Barbieri, Trends Endocrinol. Metab., 3, 30 (1992).

8. K. Gordon, F. Irianni and G.D. Hodgen, Human Reprod., 8 Suppl. 2, 204 (1993).
9. R.A. Loy, Curr. Opin. Obstet. Gy., 6, 262 (1994).
10. J.J. Nestor, Jr. and B.H. Vickery, Ann. Rep. Med. Chem., 23, 211 (1988).
11. M.J. Karten in "Modes of Action of GnRH and GnRH Analogs," W.F. Crowley, Jr. and P.M. Conn, Eds, Springer-Verlag, New York, N.Y., 1992, p. 277.
12. P.M. Conn and W.F. Crowley, Jr., Annu. Rev. Med., 45, 391 (1994).
13. M.J. Karten, W.A. Hook, R.P. Siraganian, D.H. Coy, K. Folkers, J.E. Rivier, and R.W. Roeske in "LHRH and Its Analogs, Contraceptive and Therapeutic Applications," Part 2, B.H. Vickery and J.J. Nestor, Jr., Eds., MTP Press Ltd., Lancaster, 1987, p. 179.
14. K. Gordon and G.D. Hodgen, Trends Endocrinol. Metab., 3, 259 (1992).
15. A. Ljungqvist, D.-M. Feng, P.-F.L. Tang, M. Kubota, T. Okamota, Y. Zhang, C.Y. Bowers, W.A. Hook and K. Folkers, Biochem. Biophys. Res. Commun., 148, 849 (1987).
16. S. Bajusz, V.J. Csernus, T. Janaky, L. Bokser, M. Fekete and A.V. Schally, Int. J. Peptide Protein Res., 32, 425 (1988).
17. J. Rabinovici, P. Rothman, S.E. Monroe, C. Nerenberg and R.B. Jaffe, J. Clin. Endocrinol. Metab., 75, 1220 (1992).
18. S.N. Pavlou, G.B. Wakefield, D.P. Island, P.G. Hoffman, M.E. LePage, R.L. Chan, C.A. Nerenberg and W.J. Kovacs, J. Clin. Endocrinol. Metab., 64, 931 (1987).
19. F. Haviv, T.D. Fitzpatrick, C.J. Nichols, R.E. Swenson, N.A. Mort, E.N. Bush, G. Diaz, A.T. Nguyen, M.R. Holst, V.A. Cybulski, J.A. Leal, G. Bammert, N.S. Rhutasel, P.W. Dodge, E.S. Johnson, J.B. Cannon, J. Knittleand and J. Greer, J. Med. Chem., 36, 928 (1993).
20. A. Janecka, T. Janecki, C. Bowers and K. Folkers, Amino Acids, 5, 359 (1993).
21. A. Ljungqvist, C. Bowers and K. Folkers, Int. J. Peptide Protein Res., 41, 427 (1993).
22. G.L. Plosker and R.N. Brogden, Drugs, 48, 930 (1994).
23. D.H. Coy, F. Labrie, M. Savary, E. Coy and A.V. Schally, Biochem. Biophys. Res. Commun., 67, 576 (1975).
24. J.J. Nestor, Jr, T.L. Ho, R.A. Simpson, B.L. Horner, G.H. Jones, G.I. McRae and B.H. Vickery, J. Med. Chem., 27, 320 (1984).
25. F. Haviv, T.D. Fitzpatrick, R.E. Swenson, C.J. Nichols, N.A. Mort, E.N. Bush, G. Diaz, G. Bammert, A. Nguyen, N.S. Rhutasel, H.N. Nellans, D.J. Hoffman, E.S. Johnson and J. Greer, J. Med. Chem., 36, 363 (1993).
26. A. Janecka, T. Janecki, C.Y. Bowers and K. Folkers, Int. J. Peptide Protein Res., 44, 19 (1994).
27. M. Suzuki, A. Ljungqvist, A. Janecka, C.Y. Bowers and K. Folkers, Med. Chem. Res., 1, 371 (1992).
28. A. Janecka, T. Janecki, S. Shan, C. Bowers and K. Folkers, J. Med. Chem., 37, 2238 (1994).
29. J. Rivier, J. Porter, C. Hoeger, P. Theobald, A.G. Craig, J. Dykert, A. Corrigan, M. Perrin, W.A. Hook, R.P. Siraganian, W. Vale and C. Rivier, J. Med. Chem., 35, 4270 (1992).
30. J. Pinski, T. Yano, T. Janaky, A. Nagy, A. Juhasz, L. Bokser, K. Groot and A.V. Schally, Int. J. Peptide Protein Res., 41, 66 (1993).
31. R. Deghenghi, F. Boutignon, P. Wüthrich and V. Lenaerts, Biomed. & Pharmacother., 47, 107 (1993).
32. J.J. Nestor Jr., R. Tahilramani, T.L. Ho, J.C. Goodpasture, B.H. Vickery and P. Ferrandon in "Peptides 1988," G. Jung and E. Bayer, Eds., Walter deGruyter & Co.: Berlin, 1989, p. 592.
33. J.J. Nestor Jr., R. Tahilramani, T.L. Ho, J.C. Goodpasture, B.H. Vickery and P. Ferrandon, J. Med. Chem., 35, 3942 (1992).
34. G. Flouret, K. Mahan and T. Majewski, J. Med. Chem., 35, 636 (1992).
35. A. Janecka, A. Ljungqvist, M. Suzuki, C.Y. Bowers and K. Folkers, Med. Chem. Res., 1, 376 (1992).
36. G.V. Nikiforovich and G.R. Marshall, Int. J. Peptide Protein Res., 42, 171 (1993).
37. G.V. Nikiforovich and G.R. Marshall, Int. J. Peptide Protein Res., 42, 181 (1993).
38. J.A. Janovick, F. Haviv, T.D. Fitzpatrick and P.M. Conn, Endocrinology, 133, 942 (1993).
39. F. Haviv, C.A. Palabrica, E.N. Bush, G. Diaz, E.S. Johnson, S. Love and J. Greer, J. Med. Chem., 32, 2340 (1989).
40. F. Haviv, T.D. Fitzpatrick, C.J. Nichols, E.N. Bush, G. Diaz, G. Bammert, A.T. Nguyen, E.S. Johnson, J. Knittle and J. Greer, J. Med. Chem., 37, 701 (1994).
41. K.V.R. Chary, S. Srivastava, R.V. Hosur, K.B. Boy and G. Govil, Eur. J. Biochem., 158, 323 (1986).
42. R.M. Freidinger, D.F. Veber, D.S. Perlow, J.R. Brooks and R. Saperstein, Science, 210, 656 (1980).
43. R.S. Struthers, G. Tanaka, S.C. Koerber, T. Solmajar, E.L. Baniak, L.M. Gierasch, W. Vale, J. Rivier and A.T. Hagler, Proteins, 8, 295 (1990).
44. J.E. Rivier, C. Rivier, W. Vale, S. Koerber, A. Corrigan, J. Porter, L.M. Gierasch and A.T. Hagler in "Peptides: Chemistry, Structure and Biology," J.E. Rivier and G.R. Marshall, Eds,, ESCOM, Leiden, The Netherlands, 1990, p. 33.
45. J. Rizo, S.C. Koerber, R.J. Bienstock, J. Rivier, A.T. Hagler and L.M. Gierasch, J. Am. Chem. Soc., 114, 2852 (1992).
46. J. Rizo, S.C. Koerber, R.J. Bienstock, J. Rivier, L.M. Gierasch and A.T. Hagler, J. Am. Chem. Soc., 114, 2860(1992).

47. R.J. Bienstock, J. Rizo, S.C. Koerber, J.E. Rivier, A.T. Hagler and L.M. Gierasch, J. Med. Chem., 36, 3265 (1993).
48. J. Rizo, R.B. Sutton, J. Breslau, S.C. Koerber, J. Porter, J.E. Rivier, A.T. Hagler and L.M. Gierasch in "Peptides: Chemistry, Structure and Biology," R.S. Hodges and J.A. Smith, Eds,, ESCOM, Leiden, The Netherlands, 1994, p. 766.
49. A.S. Dutta, J.J. Gormley, J.R. Woodburn, P.K.C. Paul, D.J. Osguthorpe and M.M. Campbell BioMed. Chem. Lett., 3, 943 (1993).
50. C.J. Bagatell, P.M. Conn and W.J. Bremner, Fertil. Steril., 60, 680 (1993).
51. D. Klingmüller, M. Schepke, C. Enzweiler and F. Bidlingmaier, Acta Endocrin., 128, 15 (1993).
52. H.M. Behre, B. Klein, E. Steinmeyer, G.P. MacGregor, K. Voigt and E. Nieschlag, J. Clin. Endocrinol. Metab., 75, 393 (1992).
53. H.M. Behre and E. Nieschlag, Fertil. Steril., 62, 205 (1994).
54. C.J. Bagatell and W.J. Bremner, Fertil. Steril., 62, 205 (1994).
55. G.F. Weinbauer and E. Nieschlag, Andrologia, 25, 141 (1993).
56. D. Gonzalez-Barcena, M. Vadillo Buenfil, E. Garcia Procel, L. Guerra-Arguero, I. Cardenas Cornejo, A.M. Comaru-Schally and A.V. Schally, Eur. J. Endocrinol., 131, 286 (1994).
57. L. Sommer, K. Zanger, T. Dyong, C. Dorn, J. Luckhaus, K. Diedrich and D. Klingmüller, Eur. J. Endocrinol., 131, 280 (1994).
58. L.A. Marshall, M.R. Fluker, R.B. Jaffe and S.E. Monroe, J. Clin. Endocrinol. Metab., 72, 927 (1991).
59. J.L. Andreyko, S.E. Monroe, L.A. Marshall, M.G. Fluker, C.A. Nerenberg and R.A. Jaffe, J. Clin. Endocrinol. Metab., 74, 399 (1992).
60. I. Leroy, M.F. d'Acremont, S. Brailly-Tabard, R. Frydman, J. de Mouzon and P. Bouchard, Fertil. Steril., 62, 461 (1994).
61 F. Olivennes, R. Fanchin, P. Bouchard, D. de Ziegler, J. Taieb, J. Selva and R. Frydman, Fertil. Steril., 62, 468 (1994).
62. D. Gonzalez-Barcena, M. Vadillo-Buenfil, F. Gomez-Orta, M. Garcia Fuentes, I. Cornejo Cardenas, A. Graef-Sanchez, A.M. Comaru-Schally and A.V. Schally, Prostate, 24, 84 (1994).
63. T.W. Redding, A.V. Schally, S. Radulovic, S. Milovanovic, K. Szepeshazi and J.T. Isaacs, Cancer Res., 52, 2538 (1992).
64. T. Segal-Abramson, H. Kitroser, J. Levy, A.V. Schally and Y. Sharoni, Proc. Natl. Acad. Sci. USA, 89, 2336 (1992).
65. T. Yano, J. Pinski, G. Halmos, K. Szepeshazi, K. Groot and A.V. Schally, Proc. Natl. Acad. Sci. USA, 91, 7090 (1994).
66. K.L. Raheja and A. Jordan, Regul. Toxicol. Pharmacol., 19, 168 (1994).

Chapter 19. Selective Cyclooxygenase Inhibitors

David B. Reitz and Karen Seibert
Searle Research & Development
700 Chesterfield Parkway North, St. Louis, MO 63198

Introduction - In the 1970's it was demonstrated that aspirin blocks prostaglandin production *in vivo* by inhibiting the enzyme prostaglandin synthase, or "cyclooxygenase" (COX) (1,2). COX catalyzes the rate-limiting initial step in the biosynthesis of prostaglandins (PGs) from arachidonic acid (3). Prostaglandins are produced by most cells and are found in tissues with diverse biological functions. For instance, PGs are cytoprotective in the gastrointestinal tract and are involved in maintaining normal renal function (4). PGs are also produced by numerous inflammatory cells and are often considered to be pro-inflammatory mediators of hyperalgesia, hyperemia, and edema (5-7). Like aspirin, the nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the COX enzyme, accounting for the anti-inflammatory and analgesic activity observed with these drugs *in vivo*. Unfortunately, the therapeutic usefulness of the current NSAIDs is limited by a high incidence of mechanism-based toxicity in the gastrointestinal tract (GI) that is correlated with inhibition of intestinal PG formation. NSAID-induced GI toxicity is primarily mani-fested as lesions and ulceration that can evolve into a life-threatening hemorrhage; such lesions can lead to a higher incidence of morbidity in long-term NSAID users (4). With the recent discovery of an inducible cyclooxygenase (COX-2) that appears associated with inflammation, the pharmaceutical industry now possesses a novel target for designing therapeutic agents that could provide suitable antiinflammatory and analgesic activity with greatly reduced GI or renal toxicity. Although it is likely that the literature may contain antiinflammatory compounds which are selective but unrecognized COX-2 inhibitors, this chapter describes only cyclooxygenase inhibitors whose selectivity is documented by supporting COX-1 and COX-2 enzyme data.

BIOLOGY

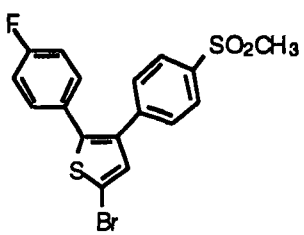
Expression and Distribution of COX *In Vivo* - The COX enzyme was first purified and subsequently cloned from sheep seminal vesicles which are an abundant source of the protein (8-12). With the availablity of the protein-encoding cDNA as well as specific antibodies, numerous studies were performed to evaluate the distribution, expression, and regulation of COX both *in vitro* and *in vivo*. A number of studies have shown that COX activity is increased in certain inflammatory states and is induced *in vitro* at a cellular level by pro-inflammatory cytokines and growth factors (13-19). For instance, incubation of dermal or synovial fibroblasts with interleukin-1 (IL-1) resulted in induction of *de novo* synthesized COX enzyme and a significant increase in the PG production by these cells; this induction was blocked by inhibitors of mRNA and protein synthesis. The potent antiinflammatory drug, dexamethasone, selectively blocked the synthesis of the "inducible" COX enzyme without altering basal COX activity in these cells (20,21). Similar experiments were carried out *in vitro* with both human monocytes and murine macrophages, demonstrating that COX activity could be "induced" by lipopolysaccharide (LPS) and selectively blocked by glucocorticoids (22). Furthermore, administration of LPS to mice resulted in a significant increase in PG production *in vivo* by peritoneal macrophages that could also be blocked by dexamethasone (23,24). These and other data suggested that there is a second, "inducible" form of the enzyme at the inflammatory site. Indeed, a second COX-expressing mRNA was identified in the chick and mouse, and subsequently in rat and human cell lines (25-29). The mRNA

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

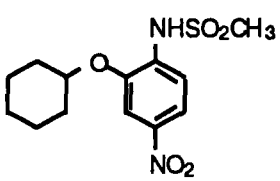
for this "COX-2" is induced both *in vitro* and *in vivo* by cytokines, growth factors, and LPS, and is selectively inhibited by dexamethasone (30-35). By contrast, the closely related mRNA encoding COX-1 is not induced, nor is its expression altered by glucocorticoids.

Identification of Selective COX-2 Inhibitors - The identification of a second COX-2 enzyme that is induced in inflammatory cells and tissues made it possible to hypothesize that selective inhibition of COX-2 would yield antiinflammatory activity without inhibiting vital COX-1-mediated PG formation in the gut or kidney (36,37). To test this hypothesis, the distribution of COX-1 and COX-2 was evaluated with specific molecular probes for each protein. COX-1 mRNA was found to be expressed in most healthy tissues and organs. Evaluation of murine tissues demonstrated that COX-1 is widely expressed in normal organs including the brain, heart, kidney, lungs, and especially abundantly in the gastrointestinal tract, while very little COX-2 mRNA was observed in healthy tissues (38, 39). By contrast, mRNA isolation from inflamed mouse tissues (e.g. carrageenan-induced foot pad edema) revealed a prodigious induction over time of COX-2 mRNA, coinciding with an increase in local PG production, edema formation, and hyperalgesic response in the animals. As anticipated, treatment of these mice with commercial NSAIDs (e.g. indomethacin) blocked carrageenan-triggered PG induction and significantly reversed the edematous and hyperalgesic responses (39).

While currently available NSAIDs display antiinflammatory and analgesic properties, their therapeutic utility is significantly limited by the attendant GI toxicity thought to be caused by concomitant inhibition of COX-1 in the gut. Two novel NSAID-like compounds arising from distinct chemical classes, Dup 697 (1) and NS-398 (2), have recently been shown in animal models to possess potent antiinflammatory and analgesic activity, without accompanying GI toxicity (40,41).



1



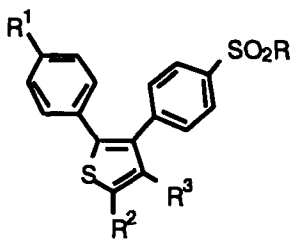
2

Such a pharmacological profile would be expected of selective COX-2 inhibitors. Recently, cloned human COX-1 and COX-2 cDNAs have been used to develop *in vitro* assays permitting the evaluation of novel compounds such as 1 and 2 with regard to selective inhibition of COX-2 (42-46). As a class, the currently available commercial NSAIDs (e.g. indomethacin, naproxen, etc.) have been found to inhibit nonselectively both COX-1 and COX-2, whereas both 1 (COX-1 IC_{50} = 0.8 μM, COX-2 IC_{50} = 0.01 μM) and 2 (COX-1 IC_{50} > 100 μM, COX-2 IC_{50} = 3.8 μM) are potent selective inhibitors of COX-2 *in vitro* (44). The commercially available NSAIDs belong to two mechanistic categories, i.e. reversible inhibitors of COX-1 (e.g. mefenamic acid), and time-dependent irreversible inhibitors (e.g. indomethacin) (47,48). Recent studies suggest that compounds 1 and 2 inhibit COX-2 in an irreversible, time-dependant manner, while weakly inhibiting COX-1 by a competitive mechanism that is independent of preincubation time (44,49).

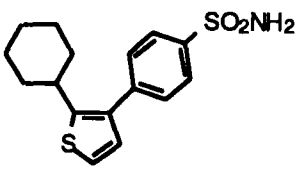
HETEROCYCLE - BASED INHIBITORS

Thiophenes - The initial reports of the 2,3-diarylthiophene 1 as a nonulcerogenic antiinflammatory agent has led to much activity in this area (40,50). Several

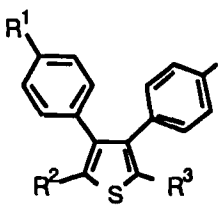
laboratories have subsequently investigated 2,3-diaryl thiophenes such as the methyl sulfones **3** and sulfonamides **4** and found them to be selective inhibitors (51,52). For example, **5** (COX-1 = 0%, COX-2 = 69% inhibition at 0.01 μM) displays selective inhibition, and it was also found to be orally active (ED_{30} = 1.4 mpk, p.o.) in rat carrageenan-induced foot pad edema (CFE) (52). The cyclohexane analog **6** (COX-1 = 2%, COX-2 = 47% inhibition at 0.01 μM) was found to have similar activity (52). The isomeric 3,4-diarylthiophenes methyl sulfones **7** and sulfonamides **8** have also been reported as selective cyclooxygenase inhibitors (52-54). Methyl sulfone **9** (COX-1 ID_{50} > 100 μM, COX-2 ID_{50} < 0.1 μM) has excellent selectivity and is orally active (30% inhibition at 10 mpk, p.o.) in the rat CFE (53) . The pyridine analog **10** was also found to be selective (COX-1 ID_{50} >10 μM, COX-2 ID_{50} = 0.2 μM) and orally active (30% inhibition at 10 mpk, p.o.) in rat CFE (53).



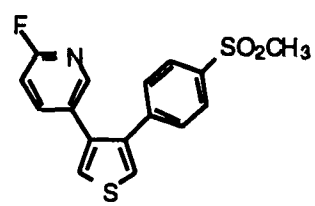
3, R = CH_3
4, R = NH_2
5, R = NH_2, R^1 = F, R^2 = R^3 = H



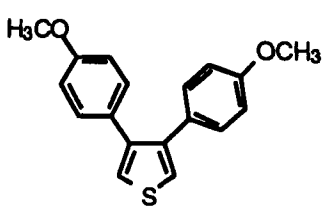
6



7, R = CH_3
8, R = NH_2
9, R = CH_3, R^1 = F, R^2 = Br, R^3 = H



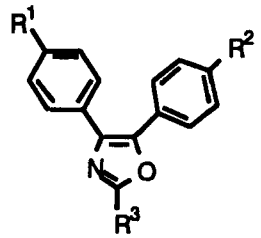
10



11

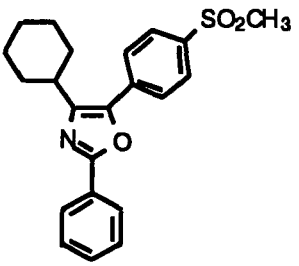
The lack of cyclooxygenase selectivity observed with 3,4-bis(4-methoxyphenyl) analog **11** (COX-1 ID_{50} = 0.3 μM, COX-2 ID_{50} = 0.8 μM) suggests that the presence of a methyl sulfone or sulfonamide moiety is required for good selectivity (53).

Oxazoles - 4,5-Diaryloxazole methyl sulfones **12** have also been reported to be selective cyclooxygenase inhibitors (55,56), displaying selectivity ratios has high as 5000:1 when the 4-methyl sulfone moiety is at the 5-position, e.g. **13** (COX-1 ID_{50} > 100 μM, COX-2 ID_{50} = 0.020 μM) and **14** (COX-1 ID_{50} =>100, COX-2 ID_{50} = 0.025 μM). The triaryl oxazole **15** (COX-1 ID_{50} >10 μM, COX-2 = 0.04 μM) was orally active (30% inhibition at 10 mpk) in rat CFE. In this series, the cyclohexane analog **16** (COX-1 ID_{50} >10 μM, COX-2 ID_{50} = 0.5 μM) proved to have reduced potency and selectivity (55,56).



12

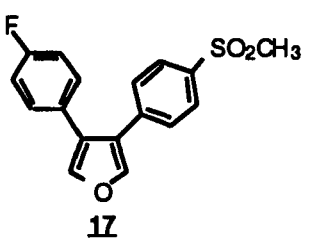
13, R^1 = F, R^2 = SO_2CH_3, R^3 = $CH_2OC_6H_4Cl$ (3)
14, R^1 = F, R^2 = SO_2CH_3, R^3 = $CH_2OC_6H_4F$ (4)
15, R^1 = F, R^2 = SO_2CH_3, R^3 = C_6H_5



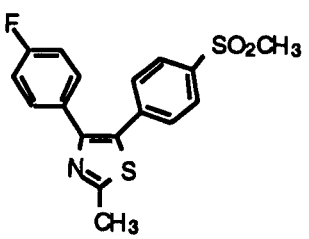
16

Furans - The 3,4-diarylfuran methyl sulfone 17 (COX-1 ID_{50} >100 μM, COX-2 ID_{50} = 1.9 μM) has been reported to be at least 10-fold less potent at inhibiting COX-2 than the corresponding thiophene analog 7 (R^1 = F, R^2 = R^3 = H) (52,53).

Thiazoles - The 4,5-diarylthiazole methyl sulfone 18 has been prepared and found to show minimal selectivity (COX-1 = 16% inhibition, COX-2 = 19% inhibition at 0.1 μM) (52).

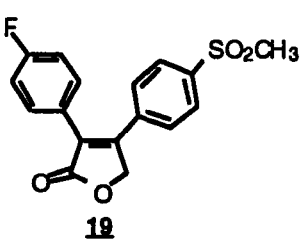


17

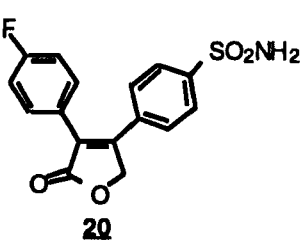


18

(5H)-Furanones - The 3,4-diaryl-5H-furanone methyl sulfone 19 (COX-1 = 9% inhibition, COX-2 = 37% inhibition at 0.1 μM) and the corresponding sulfonamide 20 (COX-1 = 12% inhibition, COX-2 = 71% inhibition at 0.1 μM) were found to have modest potency and selectivity (52). Furanone 19 was also orally active (ED_{30} = 2.8 mpk, p.o.) in rat CFE, and oral activity (ED_{30} = 0.72 mpk, p.o.) improved when tested in 1% methocel (52).

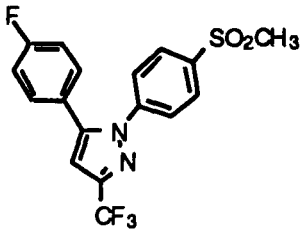


19



20

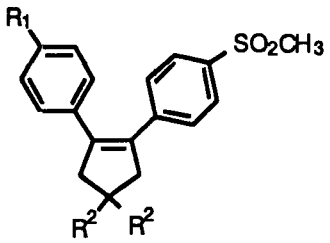
Pyrazoles - The 1,5-diaryl pyrazole **21** (SC-58125) was one of the first well documented selective cyclooxygenase inhibitors to be reported (36,39,57). Initial *in vitro* testing determined that **21** is a potent COX-2 inhibitor (COX-1 IC_{50} > 100 μM, COX-2 IC_{50} = 0.05 μM) displaying impressive selectivity (> 2000:1). Additional *in vivo* testing has established that **21** is orally active in both rat CFE (ED_{50} = 10 mpk, p.o.) and rat adjuvant-induced arthritis (AA) (ED_{50} = 0.4 mpk, p.o.). Moreover, **21** showed no indications of GI lesions when administered at 600 mpk (p.o.), consistent with its lack of COX-1 activity.

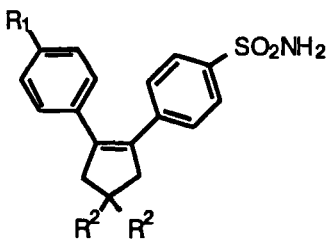


21

CARBOCYCLE - BASED INHIBITORS

Cyclopentenes - 1,2-Diarylcyclopentene methyl sulfones **22** have been reported to be orally active COX-2 inhibitors, displaying excellent potency and selectivity (58-61). For example, **23** (SC-57666) (COX-1 IC_{50} > 1000 μM , COX-2 IC_{50} = 0.026 μM) was found to be orally active in both CFE (32% inhibition at 10 mpk, p.o.) and AA (ED_{50} = 1.7 mpk, p.o.). Moreover, when **23** was administered p.o., no gastric or intestinal lesions were observed in mice (600 mpk) or in rats (200 mpk). Structure-activity relationship studies have determined that the type of substituent R^1 in **22** has little effect on COX-1 activity, except for methoxyl substitution, i.e. **24** (COX-1 IC_{50} = 9.92 μM, COX-2 IC_{50} = 0.005 μM), where a 10-fold increase in COX-1 inhibition was observed. However, the type of substituent R^1 was found to have a profound influence on COX-2 activity, e.g. **25** (COX-1 IC_{50} > 100 μM, COX-2 = 0.003 μM) and **26** (COX-1 > 100 μM, COX-2 = 0.003 μM), thereby greatly enhancing the selectivity ratio for COX-2 over COX-1 (>33,000:1). The phenyl analog **27** (COX-1 > 100 μM, COX-2 = 2.3 μM) showed a dramatic decrease in COX-2 activity, suggesting that a lipophilic substituent (R^1) at this position enhances COX-2 potency. Sulfonamide 1,2-diarylcyclopentenes **28** were found to be at least as active at inhibiting COX-2 as the methyl sulfones **22**, however, a 10 to100-fold increase in COX-1 activity was also observed (61). For example, **29** (COX-1 = 1.3 μM, COX-2 = 0.003 μM) maintained the COX-2 activity observed for **25** , while COX-1 activity increased 80-fold.





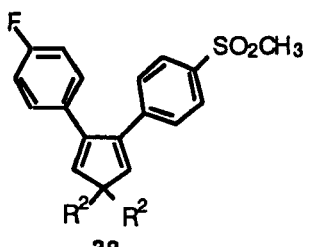
22, R^2 = H
23, R^2 = H, R^1 = F
24, R^2 = H, R^1 = OCH_3
25, R^2 = H, R^1 = Cl
26, R^2 = H, R^1 = CH_3
27, R^2 = H, R^1 = H

30, R^2 = CH_3
31, R^1 = F, R^2 = CH_3
35, R^1 = F, R^2 = CH_2F
36, R^1 = F, R^2 = CF_3
37, R^1 = F, R^2 = C_2H_5

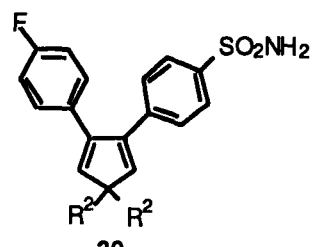
28, R^2 = H
29, R^2 = H, R^1 = Cl
32, R^2 = CH_3
33, R^2 = CH_3, R^1 = F
34, R^2 = H, R^1 = F

4-Geminally substituted cyclopentene methyl sulfones **30** were synthesized in order to probe spatial requirements of the enzyme in regions above and below the plane of the inhibitor double bond (58-61). For methyl sulfone **31** (SC-58231), a slight increase in COX-2 activity (IC_{50} = 0.015 μM *vs* 0.026 for **23**) was observed, along with a greater increase in COX-1 activity (IC_{50} = 18.3 μM *vs* >1000 μM for **23**), thus affording an inhibitor with diminished selectivity (2300:1 *vs* >38,000:1 for **23**). 4-Geminally substituted sulfonamides **32** had higher levels of COX-1 activity than the corresponding unsubstituted sulfonamides **28**, e.g. **33** (COX-1 IC_{50} = 0.49 μM, COX-2 IC_{50} = 0.008 μM) vs **34** (COX-1 IC_{50} = 4.21 μM, COX-2 IC_{50} = 0.007 μM). A decrease in COX-2 activity was found for the series **31** > **35** > **36** > **37**- *suggesting that the enzyme domain which binds this region is highly sensitive to* steric bulk (60). Oral activity in CFE for 4-geminally substituted analogs was found to be generally somewhat less than the corresponding unsubstituted analogs.

Cyclopentadienes - Cyclopentadiene inhibitors were investigated in order to determine whether completely planar carbocyclic inhibitors more closely resembling planar heterocyclic analogs such as **21** would provide improved potency and/or selectivity (61). The methyl sulfone **38** was found to be an inferior inhibitor relative to the cyclopentene **31**, due to an increase in COX-1 activity (IC_{50} = 3.68 μM *vs* 18.3 μM, respectively) and a concomitant decrease in COX-2 inhibition (IC_{50} = 0.052 μM *vs* 0.015 μM, respectively). The corresponding sulfonamide **39** (COX-1 IC_{50} = 0.027 μM, COX-2 IC_{50} = 0.003) was a very potent nonselective cyclooxygenase inhibitor with poor selectivity (61). Oral activity in CFE for cyclopentadienes was considerably less than in the corresponding cyclopentene series.

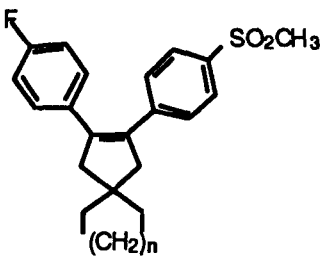


38

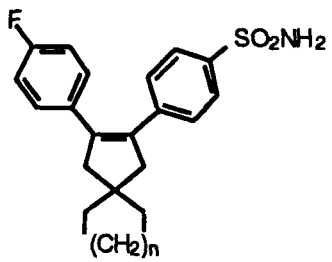


39

Spirocyclopentenes - Novel 4-spiro 1,2-diarylcyclopentene analogs have recently been reported, exemplified by methyl sulfones **40**-**43** (62,63). It was found that connecting the geminal methyl groups of the methyl sulfone **31** produced a spiro[2.4]hept-5-ene **40** (SC-58451) which was twice as potent (COX-1 IC_{50} = 5.4 μM, COX-2 IC_{50} = 0.008 μM) and half as selective (1200:1 *vs* 675:1) as the geminal dimethyl analog **31**. The methyl sulfone spiro[3.4]oct-6-ene **41** was found to have slightly more COX-2 activity (IC_{50} = 0.004 μM) than **40**, while being essentially inactive on COX-1 (IC_{50} > 100 μM). Thus, a dramatic improvement in selectivity (> 25,000:1 *vs* 675:1) was observed by expanding the size of the 4-spiro ring from cyclopropyl to cyclobutyl in this series. Further increasing the 4-spiro ring size to cyclopentyl produced **42** , and had no effect on COX-1 activity (IC_{50} > 100 μM), however, a decrease in COX-2 activity (IC_{50} = 0.062 μM) was observed. Continuing this trend, the spiro[4.5]dec-2-ene **43** (COX-1 IC_{50} > 100 μM, COX-2 IC_{50} > 100 μM) was found to be essentially inactive. 4-Spiro sulfonamides **44** (COX-1 IC_{50} = 0.33 μM, COX-2 IC_{50} = 0.003 μM) and **45** (COX-1 IC_{50} = 0.83 μM, COX-2 IC_{50} = 0.002 μM) were found to more potent but less selective than methyl sulfones **40** and **41**, respectively. These observations also support the inference (*vide supra*) that the enzyme binding domain above and below the plane of the cyclopentene double bond is highly sensitive to steric bulk. The methyl sulfone **40** has been shown to be active in rat AA (ED_{50} = 0.3 mpk, p.o.) (62).

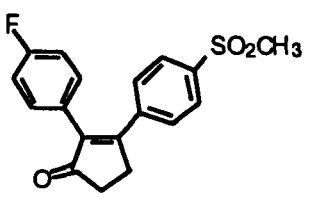


40, n = 0 **42**, n = 2
41, n = 1 **43**, n = 3



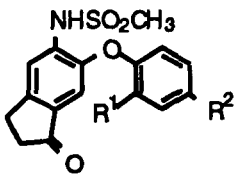
44, n = 0
45, n = 1

Cyclopenten-1-ones - The 2,3-diarylcyclopen-1-one **46** has recently been reported (52). Although **46** (COX-1 = 0% , COX-2 = 74% inhibition at 0.10 μM)showed only modest COX-2 activity, it displayed good selectivity. Considering its modest *in vitro* activity, this analog was found to have unusually good *in vivo* activity (ED_{30} = 0.43 mpk, p.o.) in the rat CFE model (52).

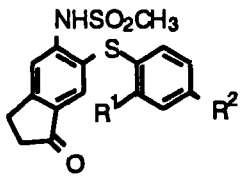


46

Indanones - There has been substantial activity in this area over the last several years, due in part to the fact that 6-phenoxy indanone **47** (Flosulide) is a well established antiinflammatory agent (64). The resemblance of this analog to **2** is noteworthy. Recent studies with **47** have established that this compound is a potent, selective inhibitor of inducible cyclooxygenase (COX-1 IC_{50} > 50 μM, COX-2 IC_{50} = 0.05 μM) and that it has considerable oral activity in rat AA (ED_{40} = 0.05 mpk, p.o.) (65,66). Additional work in this area has shown that **48** (COX-1 IC_{50} > 10 μM,



47, $R^1 = R^2 = F$
48, $R^1 = R^2 = Cl$



49, R1 = R2 = F

COX-2 IC_{50} = 0.008 μM) is more potent than **47** (67,68). Moreover, it was discovered that the 6-phenylthio indanone **49** (L-745,337) had similar enzyme activity (COX-1 IC_{50} > 10 μM, COX-2 IC_{50} = 0.050 μM) but was about twice as orally active (ED_{30} = 0.30 mpk, p.o. *vs* ED_{30} = 0.62 mpk, p.o. for **47**) in the rat CFE. More extensive *in vivo* testing in monkeys has established that **49** has better bioavailbility than does **47**. Additional studies have also demonstrated that **49** reverses hyperalgesia induced by carrageenan in rats without causing gastric ulceration at doses 100 times those causing antinociception (69).

SUMMARY AND FUTURE DIRECTIONS

The significant advances made in recent years in the discovery of selective cyclooxygenase inhibitors has been impressive. The current availability of human recombinant COX-1 and COX-2 assays used in determining enzyme selectivity has facilitated the discovery and development of selective COX-2 inhibitors as therapeutic agents for the treatment of inflammation. Since no clinical data has been reported to date, it is unknown whether selective COX-2 inhibitors will have genuine therapeutic utility in the treatment of inflammation without the gastric side-effects associated with non-selective cyclooxygenase inhibitors (NSAIDs).

References

1. J. Vane, Nature (New Biol.), 231, 232 (1971)
2. J. Smith and A. Willis, Nature (New Biol.), 231, 235 (1971).
3. P. Needleman, J. Turk, B. Jakschik, A. Morrison, and J. Lefkowith, Annu. Rev. Biochem., 55, 59 (1986).
4. M. Allison, A. Howalson, C. Torrance, P. Lee, and R. Russell, New Engl. J. Med., 327, 749 (1992).
5. I. Otterness and M. Bliven, in "Nonsteroidal Anti-inflammatory Drugs," J. Lombardino, Ed., Wiley, New York, 1985, p. 111.
6. C. Winter, E. Risley, and G. Nuss, Proc. Soc. Exp. Biol. Med., 111, 544 (1962).
7. K. Hargreaves, R. Dubner, F. Brown, C. Flores, and J. Joris, Pain, 32, 77 (1988).
8. M. Hemler, W. Lands, and W. Smith, J. Biol. Chem, 251, 5575 (1976).

9. T. Miyamoto, N. Ogino, S. Yamammoto and O. Hayaishi, J. Biol. Chem., 251, 2629 (1976).
10. F. VanDerOuderaa, M. Buyenhek, D. Nugteren, and D. VanDorp, Biochim. Biophys. Acta, 367, 315 (1977).
11. D. DeWitt and W. Smith, Proc. Natl. Acad. Sci. USA, 85, 1412 (1988).
12. J. Merlie, D. Fagan, J. Mudd, and P. Needleman, J. Biol. Chem., 263, 3550 (1988).
13. D. DeWitt, Biochim. Biophys. Acta., 1083, 121 (1991).
14. J. Bailey, B. Muza, T. Hla, and K. Salata, J. Lipid Res., 26, 54 (1985).
15. H. Sano, T. Hla, J. Maier, L. Crofford, J. Case, T. Maciag, and R. Wilder, J. Clin. Invest., 89, 97 (1992).
16. P. Jonas-Whitley and P. Needleman, J. Clin. Invest., 74, 2249 (1984).
17. A. Habenicht, M. Goerig, J. Grulcih, D. Ruthe, R. Gronwald, U. Loter, G. Schettler, B. Kommerell, and R. Ross, J. Clin. Invest., 75, 1381 (1985).
18. A. Lin, M. Bienkowski, and R. Gorman, J. Biol. Chem., 164, 17379 (1989).
19. J. Maier, T. Hla, and T. Maciag, J. Biol. Chem., 265, 10805 (1990).
20. A. Raz, A. Wyche, N. Siegel, and P. Needleman, J. Biol. Chem., 263, 3022 (1988).
21. A. Raz, A. Wyche, and P. Needleman, Proc. Natl. Acad. Sci, USA, 86, 1657 (1989).
22. J. Fu, J. Masferrer, K. Seibert, A. Raz, and P. Needleman, J. Biol. Chem., 265, 1 (1990).
23. J. Masferrer, B. Zweifel, K. Seibert, and P. Needleman, J. Clin. Invest., 86, 1375 (1992).
24. J. Masferrer, K. Seibert, B. Zweifel, and P. Needleman, Proc. Natl. Acad. Sci. USA, 89, 3917 (1992).
25. W. Xie, J. Chipman, D. Robertson, R. Erikson, and D. Simmons, Proc. Natl. Acad. Sci. USA, 88, 2692 (1991).
26. D. Kujubu, B. Fletcher, C. Varnum, W. Lim, and H. Herschman, J. Biol. Chem., 266, 12866 (1991).
27. T. Hla and K. Neilson, Proc. Natl. Acad. Sci. USA, 89, 7384 (1992).
28. J. Sirois and J. Richards, J. Biol. Chem., 267, 6382 (1992).
29. B. Kennedy, C-Culp, and W. Cromlish, Biochem. Biophys. Res. Comm., 197, 494 (1993).
30. D. Kujubu, S. Reddy, B. Fletcher, and H. Herschman, J. Biol. Chem., 268, 5425 (1993).
31. M. O'Banion, V. Winn, and D. Young, Proc. Natl. Acad. Sci. USA, 89, 4888 (1992).
32. D. Kujubu and H. Herschman, J. Biol. Chem., 267, 7991 (1992).
33. S. Lee, E. Soyoola, P. Chanmugam, S. Hart, W. Sun, H. Zhong, S. Liou, D. Simmons, and D. Hwang, J. Biol. Chem., 267, 25934 (1992).
34. T. Phillips, D. Kujubu, R. Mackay, H. Herschman, S. Russell, and J. Pace, J. Leukocyte Biol., 53, 411 (1993).
35. J. Masferrer, S. Reddy, B. Zweifel, K. Seibert, P. Needleman, R. Gilbert, and H. Herschman, J. Pharm. Exp. Ther., 270, 1340 (1994).
36. P. Isakson, K. Seibert, J. Masferrer, D. Salvemini, L. Lee, and P. Needleman, in "Proceedings of the Ninth International Conference on Prostaglandins and Related Compounds," B. Samuelsson, R. Paoletti, and P. Ramwell, Ed., Raven Press, New York, 1995, in press.
37. W. Cromlish, P. Payette, S. Culp, M. Ouellet, M. Percival, and B. Kennedy, Arch. Biochem. Biophys., 314, 193 (1994).
38. G. O'Neill and A. Ford-Hutchinson, FEBS, 330, 156 (1993).
39. K. Seibert, Y. Zhang, K. Leahy, S. Hauser, J. Masferrer, W. Perkins, L. Lee, and P. Isakson, Proc. Natl. Acad. Sci. USA, 91, 12013 (1994).
40. K. Gans, W. Galbraith, R. Roman, S. Haber, J. Kerr, W. Schmidt, C. Smith, W. Hewes, and N. Ackerman, J. Pharm. Exp. Ther., 254, 180 (1989).
41. N. Futaki, K. Yoshikawa, Y. Hamasaka, I. Arai, S. Higuchi, H. Iizuka, and S. Otomo, Gen. Pharmac., 24, 105 (1993).
42. N. Futaki, S. Takahashi, M. Yokoyama, I. Arai, S. Higuchi, and S. Otomo, Prostaglandins, 47, 55 (1994).
43. E. Meade, W. Smith, and D. DeWitt, J. Biol. Chem., 268, 6610 (1993).
44. J. Gierse, S. Hauser, D. Creely, C. Koboldt, S. Rangwala, P. Isakson, and K. Seibert, Biochem. J., 305, 479 (1995).

45. N. Futaki, I. Arai, Y. Hamasaka, S. Takahashi, S. Higuchi, and S. Otomo, J. Pharm. Pharmacol., 45, 753 (1993).
46. D. Percival, M. Ouellet, C. Vincent, J. Yergey, B. Kennedy, and G. O'Neill, Arch. of Biochem. and Biophys., 315, 111 (1994).
47. R. Kulmacz and W. Lands, J. Biol. Chem., 260, 12572 (1985).
48. L. Rome and W. Lands, Proc. Natl. Acad. Sci. USA, 72, 4863 (1975).
49. R. Copeland, J. Williams, J. Giannaras, S. Nurnberg, M. Covington, D. Pinto, S. Pick and J. Trzaskos, Proc. Natl. Acad. Sci. USA, 91, 11202 (1994).
50. S.B. Haber, U.S. Patent 4,820,827 (1989).
51. M. Matsuo, K. Tsuji, N. Konishi, and K. Nakamura, World Patent Application, WO 91/19708.
52. Y. Ducharme, J. Gauthier, P. Prasit, Y. Leblanc, Z. Wang, S. Leger, and M. Therien, World Patent Application, WO 95/00501.
53. S.R. Bertenshaw, P.W. Collins, T.D. Penning, and D.B. Reitz, World Patent Application, WO 94/15932.
54. J.Gauthier, Y. Leblanc, and P. Prasit, World Patent Application, WO 94/26731.
55. B.H. Norman, L.F. Lee, J.L. Masferrer, and J.J. Talley, World Patent Application, WO 94/27980.
56. B.H. Norman, L.F. Lee, J.L. Masferrer, and J.J. Talley, U.S. Patent 5,380,738.
57. P. Isakson, 208th National ACS Meeting, MEDI-270 (1994).
58. D.B. Reitz, J.J. Li, M.B. Norton, E.J. Reinhard, M.A. Penick, J.T. Collins, E.W. Logusch, D.J. Garland, T.S. Chamberlain, H-C. Huang, P. Isakson, K. Seibert, C. Kobold, S. Gregory, A. Veenhuizen, Y. Zhang, G. Anderson, W. Perkins, J. Casler, and C. Ponte, 208th National ACS Meeting, MEDI-271 (1994).
59. D.B. Reitz and J. Li, U.S. Patent 5,344,991.
60. D.B. Reitz, J.J. Li, M.B. Norton, E.J. Reinhard, J.T. Collins, G.D. Anderson, S.A. Gregory, C.M. Koboldt, W.E. Perkins, K. Seibert, and P.C. Isakson, J. Med. Chem., 37, 3878 (1994).
61. D.B. Reitz, J.J. Li, M.B. Norton, E.J. Reinhard, H-C. Huang, M.A. Penick, J.T. Collins, D.J. Garland, K. Seibert, C.M. Koboldt, S.A. Gregory, A. Veenhuizen, Y. Zhang, and P.C. Isakson, Med. Chem. Res., 5, 351 (1995).
62. D.B. Reitz, H-C. Huang, J.J. Li, D.J. Garland, R.E. Manning, G.D. Anderson, S.A, Gregory, C.M. Koboldt, W.E. Perkins, K. Seibert, and P.C. Isakson, BioMed. Chem. Lett., 5, 867 (1995).
63. D.B. Reitz, R.E. Manning, H-C. Huang, and J.Li, U.S. Patent 5,393,790.
64. E. Schroeder and C. Rufer, U.S. Patent 4,375,479.
65. T. Klein, R.M. Nusing, J. Pfeilschifter, and V. Ullrich, Biochem. Pharm., 48, 1605 (1994).
66. I. Wiesenberg-Boettcher, A. Schweizer, J.R. Green, K. Mueller, F. Maerki, and J. Pfeilschifter, Drugs Exptl. Clin. Res., 15, 501 (1989).
67. A.W. Ford-Hutchinson, B.P. Kennedy, P. Prasit, P.J. Vickers, C-S. Lu, C. Black, D. Guay, C.K. Lau, and P. Roy, World Patent Application, WO 94/13635.
68. P. Prasit, 208th National ACS Meeting, MEDI-272 (1994).
69. S. Boyce, C-C. Chan, R. Gordon, C-S. Li, I.W. Rodger, J.K. Webb, N.M.J. Rupniak, and R.G. Hill, Neuropharmacology, 33, 1609 (1994).

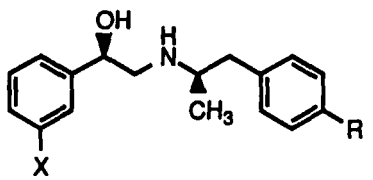
Chapter 20. β_3-Selective Adrenergic Receptor Agonists

Thomas H. Claus and Jonathan D. Bloom
Wyeth-Ayerst Research
Pearl River, NY 10965

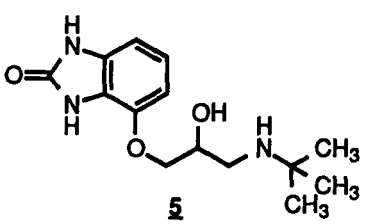
Introduction - The subdivision of β-adrenergic receptors (β-AR) into β_1- and β_2-AR has led to the development of β_1- and β_2-AR antagonists and agonists, as well as non-selective β_3–AR antagonists, that have been useful for the treatment of cardiovascular disease and asthma (1,2). The recent discovery of yet a third subtype of β-AR has led to the development of β_3-AR agonists that may prove useful in the treatment of various metabolic diseases. Two recent reviews and a monograph provide excellent, thorough discussions of β_3-AR (3-5).

Evidence for β_3-AR - The rat white adipocyte β-AR was originally classified as β_1, but later work led to the proposal that the adipocyte β-AR was a hybrid that could not be explained by the presence of separate populations of the two known subtypes (1,3-5). The hybrid nature of the adipocyte β-AR was postulated to be due to the existence of an atypical β-AR, which was first called the β_3-AR in 1983 (6).

Acceptance of the rat adipocyte β-AR as atypical was greatly aided by the reports of a series of novel phenethanolamines, including BRL 26,830 (1), BRL 28,410 (2), BRL 35,135 (3), and BRL 37,344 (4) that were more potent agonists for stimulation of adipocyte lipolysis than for either rat atrial rate and tension (β_1-AR) or guinea pig tracheal relaxation (β_2-AR) (7,8). Also, β-AR antagonists were less potent inhibitors of lipolysis than of atrium or trachea, but they had similar potencies in inhibiting lipolysis whether the agonists were novel or were older agonists such as isoproterenol (non-selective), fenoterol (β_2-selective), salbutamol (β_2-selective) or prenalterol (β_1-selective). That similar results were obtained with white or brown adipocytes suggests that the two tissues have similar atypical β-AR (7,8).



1 X = H; R = CO_2Me
2 X = H; R = CO_2H
3 X = Cl; R = OCH_2CO_2Me
4 X = Cl; R = OCH_2CO_2H



5

Characterization of the gene encoding a third subtype of β-AR, first from the human genome, and subsequently from the mouse and rat genomes, has provided a structural basis for the existence of β_3-AR (9-12). The mouse and the rat are 82% and 80% identical to the human β_3-AR. The structural similarities between the three species are consistent with their similar pharmacology when they are expressed in Chinese hamster ovary cells. The three species of β_3-AR homologues also have a similar affinity for the radioligand [^{125}I]-iodocyanopindolol that is almost two orders of magnitude lower than for β_1- and β_2-AR (9-12).

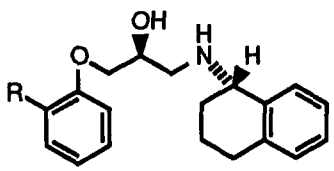
As indicated above, a common feature of atypical or β_3-AR is that they mediate responses that are insensitive to most standard β-AR antagonists. They are also more

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

responsive than β_1- or β_2-AR to novel agonists, such as **4**. A third feature is that certain antagonists of β_1- or β_2-AR are partial agonists of β_3-AR at high concentrations. One such compound is CGP 12177 (**5**) (13,14). Others are the pindolol derivatives, hydroxybenzylpindolol, cyanopindolol, and iodocyanopindolol (13,15). Using at least one of the criteria above, atypical or β_3-AR appear to be present in both white and brown adipose tissue from various species (4). However, there are significant differences in pharmacology between species (4,13).

In human adipose tissue, β-AR antagonist potencies at high concentrations were similar to those in rat white adipocytes, suggesting the presence of atypical β-AR (16). Also, **5** was more potent than **4** in stimulating lipolysis (17). Both were partial agonists and both were weakly inhibited by selective β_1- and β_2-AR antagonists. These properties plus the identification of β_3-AR mRNA in the same tissue supports the presence of β_3-AR in human adipose tissue. However, it is apparent that **4** is much less potent in human adipocytes than in rat adipocytes, and even has been reported to be ineffective (13,18).

β_3-AR Selectivity - The selectivity of β_3-AR agonists has typically been determined by functional assays in adipocytes or colon, atrium and trachea or uterus. More recently, a series of β_3-AR agonists has been ranked by their ability to stimulate cyclic AMP production in Chinese hamster ovary cells that have been transfected with the human β_1-, β_2- or β_3-AR (19,20).



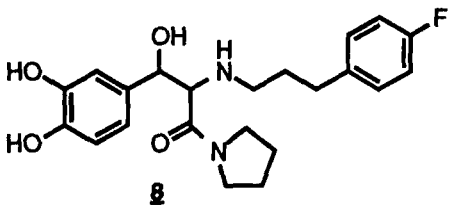
6 R = allyl (hydrochloride)
7 R = Et (oxalate)

β_3-AR Antagonists - Two aryloxypropranol-aminotetralines, SR 58894A (**6**) and SR 59203A (**7**) have recently been reported to be selective antagonists for the β_3-AR (21). *In vitro*, **6** was 15- and 46-fold more potent for inhibition of rat colon than for guinea pig atrium or trachea, respectively, while **7** was 48- and 141-fold more selective. *In vivo*, **7** was 15- and 13-fold more potent for colon motility than for tachcardia and bronchodilation, respectively.

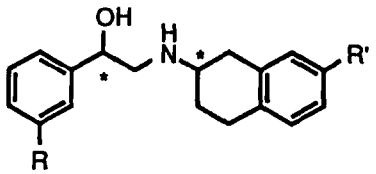
β_3-AR TISSUE DISTRIBUTION

Gastrointestinal Tract - Like the rat adipocyte β-AR, classification of the rabbit jejunum β-AR as a β_1-AR was questioned long before the advent of β_3-AR agonists (22). Early reports on gastrointestinal tract β-AR of other species also suggested the presence of a β-AR that was different from the classical β-AR (3-5). Since the introduction of the β_3-AR agonists, the same criteria for adipose tissue has been used to identify β_3-AR throughout the gastrointestinal tract of several species. In guinea pig ileum, propranolol and other antagonists were unable to antagonize all of the isoproterenol-induced relaxation (23,24). The order of potency of agonists for the propranolol-resistant responses was **4** > isoproterenol > norepinephrine which suggests that they are mediated by β_3-AR. The relaxation stimulated by SM-11044 (**8**) was also resistant to propranolol but was antagonized by cyanopindolol (25). The potency of **8** was 4.3- and 230-fold greater for ileum than for trachea and atrium, respectively, and was also 40-fold greater than for stimulation of rat white adipocyte lipolysis (25). The guinea pig gastric fundus and cecum also appear to have β_3-AR (26,27).

The presence of atypical β-AR in rat colon has been confirmed using novel phenylethanol-aminotetralines such as **9**-**13** (28). SR 58611 (**9**) was 142- and >8500-fold more potent in the colon than in trachea or atrium, respectively (28). Recently, SR-59062 (**11**) was shown to be 4 times more potent and selective than **9** (29). Propranolol and alprenolol were relatively ineffective antagonists toward **9**. There was a good correlation between the potency of phenylethanolaminotetralines toward the



8



	R	R'
9	Cl	OCH_2CO_2Et (R,S)
10	H	OH (R,R)
11	Cl	$(CH_2)_2CO2Et$ (R,R)
12	Cl	OCH_2CO_2Et (R,R and S,S)
13	Cl	OH (R,R)

colon and for rat white adipocyte lipolysis which suggests that the β_3-AR are similar in the two tissues (30). Propranolol was relatively ineffective against isoproterenol and **4** in rat distal colon, gastric fundus, and jejunum (31-33). The potency or intrinsic activity of a series of β-AR agonists to relax rat gastric fundus correlated well with their ability to activate recombinant rat β_3-AR, providing evidence that the atypical β-AR in the fundus is the β_3-AR (34). Also, β_3-AR mRNA was identified in rat gastric fundus (34). β_3-AR also appear to stimulate acid secretion by rat stomach, bicarbonate secretion by rat cecum and esophageal smooth muscle relaxation (35-37).

Bronchopulmonary System - Methacholine-induced albumin transport in ferret tracheal epithelium was more potently stimulated by **4** than by prenalterol or salbumatol and was weakly antagonized by ICI 118,551 (38). Both **4** and **7** modulated constriction of guinea pig bronchi in response to electrical field stimulation (39,40). β-AR antagonists were either weakly effective or ineffective against the β_3-AR agonists. However, neither **4** nor **7** induced relaxation of guinea pig bronchi induced by histamine (41). The same agonists were also ineffective in human and sheep bronchi (41). β_3-AR also are involved in stimulation of ciliary motility in rabbit and canine bronchial epithelium (42,43).

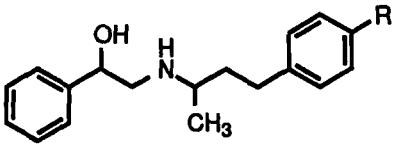
Cardiovascular and Other Systems - Atypical β-AR may play a role in cardiac tissue based on the stimulation of guinea pig sinoatrial node by pindolol-related agonists, the effects of which are resistant to β_1- and β_2-blockade (44,45). In normal dogs, **4** and **5** caused decreased blood pressure and increased heart rate, but in sinaortic denervated dogs they only decreased blood pressure (46). Neither **4** nor **5** stimulated adenylyl cyclase in canine cardiac membranes. Thus, the increased heart rate is due to baroreflex mechanisms and not to the presence of cardiac β_3-AR in dogs. The hypotensive effect of **4** in dogs appears to be due to peripheral vasodilation, primarily of skin and fat (47). β_3-AR may be found in rat common carotid arteries, since **4** and **5** relaxed norepinephrine-induced contractions (48). In man, nadolol was unable to prevent the isoproterenol-induced increase in systolic blood pressure and left ventricular stroke distance, implying the presence of β_3-AR (49).

β_3-AR appear to be found in adult, but not in newborn rat liver (50,51). They may also occur in rat soleus muscle, bovine adrenal medulla, rodent brain and guinea pig eosinophils (52-55). They have been identified in SK-N-MC cells, a human neuroblastoma cell line (56). They may play a role in extrarenal potassium disposal via activation of the Na^+- K^+-pump (57).

β_3-AR AGONISTS AS ANTIOBESITY AND ANTIDIABETES AGENTS

Rationale - The discovery that brown adipose tissue lipolysis is regulated by β_3-AR has led to the hypothesis that thermogenesis in this tissue is also regulated by the same receptors and that β_3-AR agonists may be useful as antiobesity agents. Thermogenesis, the major function of brown adipose tissue, is initiated by sympathetic release of norephinephrine which acts predominately via β-AR to cause increased lipolysis. The released fatty acids act as both substrate for β-oxidation and to overcome the restraint on respiration exerted by purine nucleotides, such as GDP, binding to uncoupling protein, a protein unique to brown adipose tissue. Activation of this protein dissipates the hydrogen gradient across the inner mitochondrial membrane, and the energy of that gradient is converted to heat rather than ATP (4,5,58). Brown adipose tissue may also play an important role in glucose homeostasis and β_3-AR agonists may be useful as antidiabetic agents as well (5,58).

Phenethanolamines- A series of phenethanolamines displaying antiobesity and antidiabetic potential began appearing in the patent literature in the late 1970s. These compounds are mimics of the hormones epinephrine and norepinephrine, and all active compounds to date have retained the requisite ethanolamine substructure. Selectivity for the β_3-AR has primarily been conferred by addition of an arylalkyl group on the amine portion of the molecule. It is in this area of the molecule that the greatest structural variation is found.

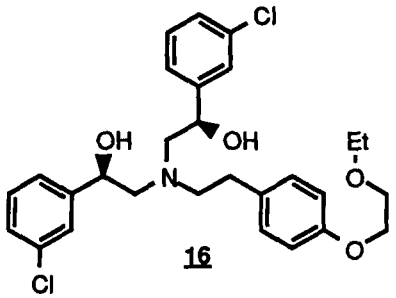


14 R = OH
15 R = $CONH_2$

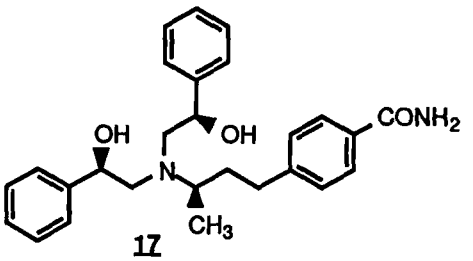
One of the earliest described compounds in this series is LY 79771 (14) (59,60). This compound displayed several properties consistent with use as an antiobesity agent, including reduced weight gain (reversible) and increased body temperature in several animal models of obesity (61,62). No effect was seen in normal animals. The development of 14 was discontinued (63). Substitution of a carboxamido group for the aryl hydroxyl group led to LY 104119 (15) which stimulated cAMP production and lipolysis in adipose tissue, and increased CO_2 and heat production and significantly decreased weight gain in mouse and rat models of obesity (64,65). This compound has been reviewed in this journal (66).

A novel tertiary amine, Ro 16-8714 (16) was shown to possess significant antiobesity/antidiabetic properties including increased oxygen consumption in rat and mouse models of obesity, stimulation of oxidation of both fat and carbohydrates and inhibition of weight gain (67-69). In human trials there was a demonstrated increase in resting metabolic rate, however, this was accompanied by a dose dependent increase in heart rate and systolic blood pressure , and development was discontinued (70,71). Minor structural modification of 16 led to Ro 40-2148 (17) (72). Like its predecessor, 17 increased oxygen consumption in several species (73). Increased lipid oxidation was shown to be responsible for the increase in metabolism, and only a small increase in heart rate was noted in rats. 17 displayed greatly enhanced β_3 selectivity, being at least 20-fold more potent in inducing thermogenesis than heart rate increase. Genetically obese rats lost weight, accompanied by reductions in serum and liver lipid. Increased BAT weight and protein content were also noted. In human clinical trials, a change in energy expenditure (mean 7.9%) was noted only at the highest dose of 800

mg (74). The compound was well tolerated and no increase in heart rate or blood pressure was observed.



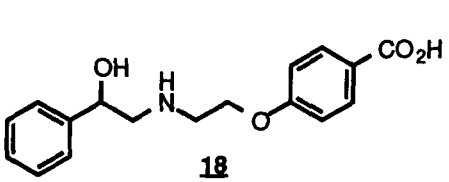
16



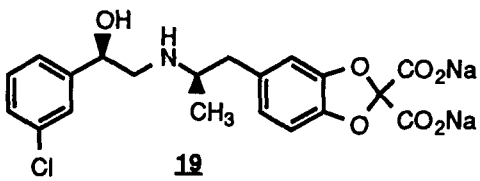
17

Methyl ester 1 (the active metabolite being acid 2) has been intensely studied as a potentially β_3-selective antiobesity agent (8,66,75,76). *In Vitro*, 2 has a 20-fold selectivity for lipolysis (a β_3 process) over atrial stimulation (a β_1 process), and an eight-fold selectivity for lipolysis over tracheal stimulation (a β_2 process). In genetically obese mice, 1 produced a significant and sustained increase in metabolic rate and a decrease in weight gained with no effect on food consumption. BAT hypertrophy was noted following repeat dosing and improved sensitivity to insulin was noted in three animal models of Type II diabetes (8,77,78). Human clinical trials produced conflicting results, showing either modest or no weight loss, depending on the protocol of the study (79-84). Although there was no significant increase in heart rate, tremor and shaky hands were problematic, occurring in 25-85% of patients (79,84-86). Thus, the development of 1 was discontinued (86).

Aryloxyacetate 3 (the carboxylic acid metabolite 4 being the active species) shows an improved selectivity profile over its predecessor 1 (400x β_3/β_1 and 20X β_3/β_2) (8,76,87,88). As was the case with 1, 3 significantly increased the metabolic rate in obese mice while decreasing the weight gain (fat only) despite no change in food intake. Chronic dosing of obese mice and rats led to an improvement in both hyperglycemia and hyperinsulinemia at a dose that did not affect body weight (89-91). In obese humans, treatment with 3 resulted in an improvement in glucose tolerance as well as hyperinsulinemia; however, no change in body weight was noted during the 10 day trial (90,92). While no increase in heart rate was noted, there was a transient tremor (fingers) noted in 40% of the subjects. Another trial involving obese Type II diabetics showed that treatment with 3 produced a 91% increase in insulin-mediated glucose disposal, while being generally well tolerated (89, 90).



18



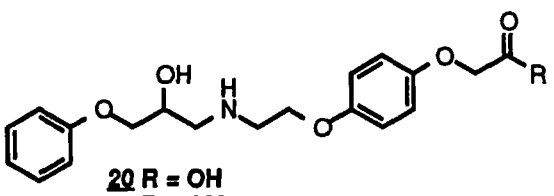
19

ZD2079 (18) stimulated rat white adipocyte lipolysis with an EC_{50} of 0.35 μM while 10 μM had no effect on guinea pig atrium and only decreasing tracheal tone by 25% (93). In conscious rats, 18 increased oxygen consumption in a dose-related manner (ED_{50}=0.4mg/kg po) and produced a similar dose-related increase in GDP binding to brown adipose tissue mitochondria (ED_{50}=0.5 mg/kg po). Heart rate was only increased at oral doses (>5 mg/kg) greater than those required for a maximum increase in thermogenic activity. In *in vivo* models of β_2-AR agonist activity, there was no effect on blood potassium levels in dogs at 10 mg/kg, and only very little effect on cat soleus muscle tremor (ED_{50} >20 mg/kg i.v.) (93). Single oral doses of 600 mg of 18

given to human volunteers had no effect on plasma potassium, electrocardiogram, blood pressure or pulse. There was an increase in free fatty acids at 2 h after dosing, consistent with the effect on lipolysis (94).

CL 316,243 (19) stimulated rat white adipocyte lipolysis with an EC_{50} of 0.3 nM, and has a >30,000 to 1 β_3-to-β_1-AR selectivity ratio and a 10,000 to 1 β_3-to-β_2-AR selectivity ratio in *in vitro* (95-97). Furthermore, 19 stimulated cAMP production in CHO cells expressing the human β_3-AR with an EC_{50} of 68 nM and bound to the receptor with an affinity of 14 mM. No binding to CHO cells expressing β_1-or β_2-AR was observed at 0.1 mM (97). 19 is the most selective β_3 agonist yet described.

In obese (ob/ob) mice, 19 reduced their hyperglycemia to the euglycemia of their lean littermates, decreased insulin levels and decreased weight gain despite increased food consumption (98). The decreased weight was due to loss of fat only. Both insulin and glucose tolerance were improved, suggesting improved insulin sensitivity. Thermogenesis was increased by 45% (98). Similar effects were observed in yellow KK mice, in monosodium-L-glutamate-induced obesity, and in young rats eating a high fat diet to induce obesity (99-101). In the latter model, interscapular BAT was markedly hypertrophied, with 3-to-4-fold increases in uncoupling protein and cytochrome oxidase, but the amount of uncoupling protein per mitochondrion was unchanged (101). White adipose tissue depots weighed less and had fewer cells (lower DNA content), and some multilocular adipocytes appeared in these normally unilocular depots. Subsequently, it has been shown that the marked thermogenic effect of 19 can not only retard, but also reverse established diet-induced obesity in rats eating a high fat diet (102,103). 19 was well tolerated at doses as high as 1500 mg/day in healthy human volunteers. No drug-related side effects attributable to β_1- or β_2-receptor stimulation were noted (96).

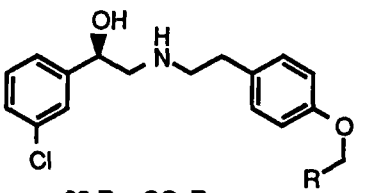


20 R = OH
21 R = OMe
22 R = $NHCH_2CH_2OMe$ (S Isomer)

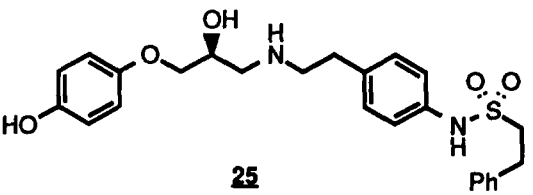
Phenoxypropanolamines - The metabolite 20 (ICI 201651) of methyl ester 21 (ICI 198157) stimulated oxygen consumption in rat BAT cells (EC_{50} = 19 nM) while having little effect on guinea pig atrial rate or tracheal relaxation (104,105). Genetically obese rats treated with 21 had a reduced weight gain despite no change in food intake. This decreased weight gain was associated with a decrease in the fat composition of the rats (105). A relatively short half-life in rats and dogs led to the preparation of 22 (ZD7114, formerly ICI D7114) (22) (106, 107). The methyl ester was replaced by a methoxyethyl amide group in an attempt to retard hydrolysis. 22 demonstrated similar *in vitro* selectivity, and stimulated oxygen consumption in three species with no effect on heart rate (108,109). Antiobesity effects were noted in Zucker rats and dogs with no associated change in food consumption (108, 109). In human trials, however, 22 showed no thermogenic activity in either obese or lean volunteers (110,111). This lack of efficacy was attributed to differences in β_3-AR in different species (112).

New Compounds - A des-methyl analog of 3 (23) appeared in a recent patent (113). Like its predecessor, 23 demonstated β_3 selectivity having an EC_{50} of 41.7 nM for lipolysis and Ki values of 7610 nM and 3656 nM for rat β_1-and β_2- receptors, respectively. Isosteric phosphonates (24) have also been claimed as selective β_3 agonists (114). A series of novel phenylsulfonamides, represented by 25, claims to be useful for treatment of diabetes and obesity, as well as inflammation, depression and

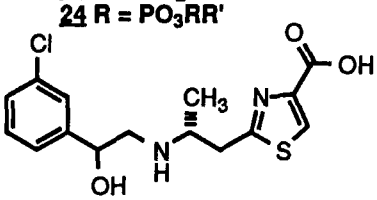
diseases of the gut (115). A series of heterocyclic phenethanolamines has been described, exemplified by thiazole **26** (116). These compounds are claimed to be useful for the treatment of diabetes, obesity and gastric hypermotility, with possibile indications for a variety of other disorders. Close relatives of **21** having a hydrolytically inert carbonyl group have recently been claimed (117). Exemplified by **27**, this class showed activity as thermogenic agents in rats (ED_{50} 0.01-10 mg/kg) and is described as lacking cardiac side effects. Fixing the methyl group of **3** into a seven-membered ring gave rise to **28**, potentially useful in treating GI disorders and obesity (118). **28** had an IC_{50} of 0.68 nM for rat distal colon (β_3) versus 200 nM for uterine smooth muscle (β_2). Recently a series of novel heterocycles represented by benzofuran **29** has been claimed to have antidiabetic and anorectic activity (119).



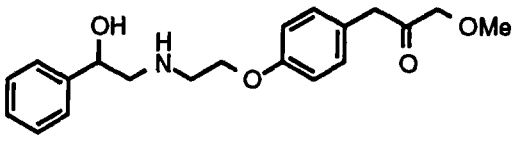
23 R = CO_2R
24 R = PO_3RR'



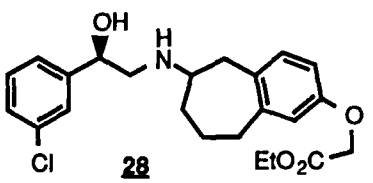
25



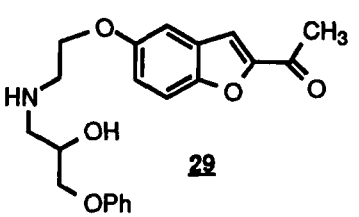
26



27



28



29

OTHER POTENTIAL THERAPEUTIC AREAS

Gut Motility - Compounds **10**-**12** were tested *in vivo* for their ability to inhibit the spontaneous motility of rat proximal colon (120). The compounds caused dose-dependent inhibition with ED_{50} values of 210, 92, and 19 μg/kg i.p., respectively, which were antagonized by alprenolol. The minimally effective doses required to increase heart rate were 2-to-5-fold higher than the ED_{50} values for inhibition of colon motility. This class of compounds may be useful for intestinal hypermotility disorders.

Depression and Other Areas - The antidepressant effects of **9** were studied in five rodent models of depression (54). The compound was effective in three models at minimally effective doses of 0.1-0.3 mg/kg i.p. Its effects were not blocked by selective β_1- and β_2-AR antagonists, but were by high doses of propranolol and alprenolol. It also has been reported that obese patients treated with **1** had a significant reduction in the somatic symptoms of anxiety that appeared to be independent of weight loss (121). β_3-AR agonists may also be useful in the treatment of NSAID-induced ulcers (122). Several other potential therapeutic areas for β_3-AR agonists have been described, based on *in vitro* effects, including inflammatory airway diseases such as asthma (5).

Conclusion - The clinical utility of β_3-AR agonists will depend on both their efficacy and on their selectivity. The presence of significant β_1- and β_2-AR related side-effects has been responsible for stopping development of several compounds and highlights the importance of optimizing β_3-selectivity against human receptors. Whether any of these agonists prove to be useful in the treatment of human diseases must await the results of clinical trials.

References

1. A.M. Lands, A. Arnold, J.P. McAuliff, F.P. Luduena, T.G. Brown, Nature, 214, 597 (1967).
2. A.M. Lands, F.P. Luduena, H.J. Buzzo, Life Sci., 6, 2241 (1967).
3. R. Howe, Drugs Fut., 18,529 (1993).
4. J.R.S. Arch, A.J. Kaumann, Med. Res. Rev., 13, 663 (1993)
5. D.E. Goldberg, W.H. Frishman, "Beta3-Adrenergic Agonism", Futura Publishing, Armonk, N.Y., 1995
6. S. Tan, P.B. Curtis-Prior, Int. J. Obesity, 7, 409 (1983)
7. C. Wilson, S. Wilson, V. Piercy, M.V. Sennitt, J.R.S. Arch, Eur. J. Pharmacol., 100,309 (1984).
8. J.R.S. Arch, A.T. Ainsworth, M.A. Cawthorne, V. Piercy, M.V. Sennitt, V.E. Thody, C. Wilson, S. Wilson, Nature, 309, 163 (1984).
9. L.J. Emorine, S. Marullo, M-M. Briend-Sutren, G. Patey, K. Tate, C. Delavier-Klutchko, A.D. Strosberg, Science, 245, 1118 (1989).
10. C. Nahmias, N. Blin, J-M. Elalouf, M.G. Mattei, A.D. Strosberg, L.J. Emorine, EMBO J., 10, 3721 (1991).
11. J.G. Granneman, K.N. Lahners, A. Chaudhry, Mol. Pharmacol., 40, 895 (1991).
12. P. Muzzin, J-P. Revelli, F. Kuhne, J.D. Gocayne, W.R. McCombie, J.G. Venter, J-P. Giacobino, C.M.Fraser, J. Biol. Chem., 226, 24053 (1991).
13. M. Lafontan, Cell. Signal., 6, 363 (1994)
14. I. Van Liefde, A. Van Witzenberg, G. Vauqelin, J. Pharmacol. Exp. Ther. 262, 552 (1992).
15. G. Engel, D. Hoyer, R. Berthold, H. Wagner, N-S Arch. Pharmacol., 317, 277 (1981)
16. J. Zaagsma, Ch. Hollenga, "Distribution and Function of Atypical β_3-Adrenoceptors" in Adrenoceptors: Structure, Mechanisms, Function, E. Szabadi, C.M. Bradshaw, Eds., Birkhäuser Verlag,Basel, p.47, (1991).
17. F. Lönnqvist, S. Krief, A.D. Strosberg, B. Nyberg, L.J. Emorine, P. Arner, Br. J. Pharmacol., 110, 929 (1993).
18. I. Van Liefde, A. Van Ermen, G. Vauquelin, Pharmacol. Lett. 54, 209 (1994).
19. N. Blin, L. Camoin, B. Maigret, A.D. Strosberg, Mol. Pharmacol., 44, 1094 (1993).
20. N. Blin, C. Nahmias, M.F. Drumare, A.D. Strosberg, Br. J. Pharmacol., 112, 911 (1994).
21. L. Manara, D. Badone, M. Baroni, G. Boccardi, R. Cecchi, T. Croci, A. Guidice, U. Guzzi, G. Le Fur, "Aryloxypropranolaminotetralins are the first selective antagonists for atypical (β_3) β-adrenoceptors", in Pharmacology of Adrenoceptors, King of Prussia, July 21-23 (1994).
22. R.F. Furchgott, "A classification of adrenoceptors (adrenergic receptors). An evaluation from the stand point of receptor theory. In Catecholamines, H.Blaschko, E. Muscholl, Eds. Springer, Berlin, p.283, 1972.
23. R.A. Bond, D.E. Clarke, Br. J. Pharmacol., 91, 683 (1987).
24. R.A. Bond, D.E. Clarke, Br. J. Pharmacol., 95, 723 (1988).
25. T. Sugasawa, M. Matsuzaki, S. Morooka, N. Foignant, N. Blin, A.D. Strosberg, Eur. J. Pharmacol., 216, 207 (1992).
26. R.A. Coleman, L.H. Denyer, K.E. Sheldrick, Br. J. Pharmacol., 87, 40P (1990).
27. K. Koike, I. Takayanagi, M. Muramatsu, S. Ohki, T. Horinouchi, Jpn. J. Pharmacol., 66, 213 (1994).
28. A. Bianchetti, L. Manara, Br. J. Pharmacol., 100, 831 (1990).
29. D. Badone, U. Guzzi, Bioorg. Med. Chem. Lett., 4, 1921 (1994).
30. A. Bianchetti, L. Manara, A.M. Codegoni, A. Bizzi, Eur. J. Pharmacol., 183, 2196 (1990).
31. D.P. McLaughlin, A. MacDonald, Br. J. Pharmacol., 101, 569 (1990).
32. D.P. McLaughlin, A. MacDonald, Br. J. Pharmacol., 103, 1351 (1991).
33. A. MacDonald, I.J. Forbes, D. Gallacher, G. Heeps, D.P. McLaughin, Br. J. Pharmacol., 102, 167P (1991).
34. M.L. Cohen, J.G. Granneman, A. Chaudhry, K.W. Schenck, D.J. Cushing, A.D. Palkowitz, J. Pharmacol. Exp. Ther. 272, 446 (1995).
35. P. Canfield, P. Paraskeva, Br. J. Pharmacol., 106, 583 (1992).
36. P. Canfield, T. Abdul-Ghaffar, Eur. J. Pharmacol., 216, 293 (1992).
37. R.E.P. deBoer, F. Brouwer, J. Zaagsma, Br. J. Pharmacol., 110, 442 (1993).
38. S.E. Webber, M.J. Stock, Br. J. Pharmacol., 105, 857 (1992).

39. S. Itabashi, T. Aikawa, K. Sekizawa, H. Sasaki, T. Takishima., Eur. J. Pharmacol., 218, 187 (1992).
40. C.A.E. Martin, E. Naline, L. Manara, C. Advenier, Br. J. Pharmacol., 110, 1311 (1993).
41. C.A.E. Martin, E. Naline, H. Bakdach, C. Advenier, Eur. Respir. J., 7, 1610 (1994).
42. K. Takeyama, J. Tamaoki, A. Chiyotani, N. Sasaki, T. Kanemura, K. Konno, Kokyu to Junkan-Resp & Circ, 41, 993 (1993).
43. J. Tamaoki, A. Chiyotani, N. Sasaki, K. Konno, Life Sci., 53, 1509 (1993).
44. A.J. Kaumann, TIPS, 10, 316 (1989).
45. M. Walter, H. Lemoine, A.J. Kaumann, N-S. Arch. Pharmacol., 327, 159 (1984).
46. G. Tavernier, J. Galitzky, A. Bousquet-Melou, J.L. Montastruc, M. Berlan, J. Pharmacol. Exp. Ther., 263, 1083 (1992).
47. Y-T. Shen, H. Zhang, S.F. Vatner, J. Pharmacol. Exp. Ther. 268, 466 (1994).
48. M.A. Oriowo, Br. J. Pharmacol., 113, 699 (1994).
49. N.M. Wheeldon, D.G. McDevitt, B.J. Lipworth, Quart. J. Med., 86, 255 (1993).
50. M.P. Sanghani, P.J. Scarpace, J. Geront.: Biol. Sci., 49, B60, (1994).
51. N. Fraeyman, A. Van Ermen, E. Van de Velde, P. Vanscheeuwijck, Biochem. Pharmacol., 44, 2333 (1992).
52. R.A.J. Challis, B. Leighton, S. Wilson, P.L. Thurlby, J.R.S. Arch, Biochem. Pharmacol., 37, 947 (1988).
53. K. Carlson, J. R. Tobin, M.J. Breslow, Eur. J. Pharmacol., 263, R1 (1994).
54. J. Simiand , P.E. Keane, J. Guitard, X. Langlois, N. Gonalons, P. Martin, A. Bianchetti, G. Le Fur, P. Soubrie, Eur. J. Pharmacol., 219, 193 (1992).
55. T. Sugasawa, S. Morooka, Agents Action, 37, 232 (1992).
56. T.A. Esbenshade, C. Han, T.L. Theroux, J.G. Granneman, K.P. Minneman, Mol. Pharmacol., 42, 753 (1992).
57. M. Reverte, M.J. Garcia-Barrado, F.J. Hernandez-Garcia, J. Moratinos, J. Autonomic Pharmacol., 13, 227 (1993).
58. J. Himms-Hagen, "Brown Adipose Tissue Metabolism" in Obesity, P. Björntorp, B.N. Brodoff, Eds., Lippincott, Philadelphia, p.15 (1992).
59. J. Mills, K.K. Schmiegel W.N. Shaw, Eur. Patent 7204 (1979).
60. Drugs of the Future,8, 923 (1983). Update- Ibid, 11, 877 (1984).
61. W.N. Shaw, K.K. Schmiegel, T.T. Yen, R.E. Toomey, D.B. Meyers, J. Mills, Life Sci. 29, 2091 (1981).
62. T.T. Yen, Int. J. Obesity, 8, 69 (1984).
63. Pharmaprojects, 3 (1983)
64. J. Mills, K.K. Schmiegel, R.R. Tuttle, US Patent Applic. 4,391,826 (Filed 1979)
65. T.T. Yen, M.M. McKee, N.B. Stamm, Int. J. Obesity, 8, 65 (1984).
66. J. Triscari, J. Tilley S. Hogan, Ann. Rep. Med. Chem., 23, 191 (1988).
67. L.Alig, M. Mueller, Eur. Patent Applic. EP 101069 (Filed 1982).
68. M.K. Meier, L. Alig, M.E. Burgi-Saville M. Muller, Int. J. Obesity, 8(Suppl. 1), 215 (1984)
69. P. Ferré, L. Pénicaud, Y. Hitier, M. Meier J. Girard, Metabolism, 41, 180 (1992).
70. Y.C. Henny, A. Buckert, M. Meylan, E. Jéquier, J.P. Felber, Int. J. Obesity, 11, 473 (1987)
71. E. Jéquier, R. Munger, J.-P. Felber, Amer. J. Clin. Nut., 55, 249S (1992).
72. L. Alig E. Mueller Eur. Patent Applic. EP 198412 (Filed 1985)
73. M.K. Meier, D. Blum-Kaelin, M. Gerold, D. Isler, M. Mueller in "Obesity in Europe 88. Proceedings of the 1st European Congress on Obesity," P. Bjorntorp S. Rossner Eds. John Libbey, London, 1989, p. 329.
74. R. Munger, A. Bückert, E. Jéquier, J.-P. Felber, Diabetes, 39(Suppl. 1), Abstr. 1100 (1990)
75. Drugs Fut., 10, 188 (1985).
76. E. R. Larson, D.A.Clark, R.W. Stevenson, Ann. Rep. Med. Chem., 25, 205 (1990).
77. J.R.S. Arch, A.T. Ainsworth, Amer. J. Clin. Nutr., 38, 549 (1983)
78. J.R.S. Arch, A.T. Ainsworth, R.D.M. Ellis, V. Piercy, V.E. Thody, P.L Thurlby, C. Wilson, S. Wilson, P. Young, Int. J. Obesity, 8(Suppl. 1), 1 (1984).
79. C.A. Zed, G.S. Harris, P.J. Harrison, G.H. Robb, Int. J. Obesity, 9, 231 (1985).
80. A.A. Connacher, R.T. Jung, P.E.G. Mitchell, Int. J. Obesity, 11, 307A (1987).
81. A.A. Connacher, R.T. Jung, P.E.G. Mitchell, Brit. Med J., 296, 1217 (1988)
82. A.A. Connacher, W.M. Bennett, R.T. Jung, Amer. J. Clin. Nutr, 55, 258S (1992).
83. B.J. Chapman, D. Farquahar, S. Galloway, G.K. Simpson, J.F. Munro, Int. J. Obesity, 9, 230 (1985).
84. B.J. Chapman, D. Farquahar, S. McL. Galloway, G.K. Simpson, J.F. Munro, Int. J. Obesity, 12, 119 (1988).
85. J.F. Munro, B.J. Chapman, G.H. Robb, C. Zed, in "Recent Advances in Obesity Research, V." E.M. Berry, S.H. Blondheim, H.E. Eliahou, E. Shafrir, Eds., John Libbey, London, 1987, p. 155.
86. IMS - Marketletter, October 22, 1990.
87. J. R. Colca, S.P. Tanis, Ann. Rep. Med. Chem., 27, 219 (1992)
88. B.C.C. Cantello, S.A. Smith, Drugs of the Future, 16, 797 (1991).
89. S.A. Smith, M.V. Sennitt, M.A. Cawthorne in "New Anti-Diabetic Drugs," C.J. Bailey P.R. Flatt, Eds., Smith-Gordon, London, 1990, p. 177.

90. M.A. Cawthorne, M.V. Sennitt, J.R.S. Arch, S.A.Smith, Amer. J. Clin. Nutr, 55, 252S (1992).
91. M.A. Cawthorne, P. Young, S.A. Smith, Diabetes, 35 (Suppl. 1), Abstr. 263 (1986).
92. T.H. Mitchell, R.D.M. Ellis, S.A. Smith, G. Robb, M.A. Cawthorne, Int. J. Obesity, 13, 757 (1989).
93. T.L. Grant, R.M. Mayers, S.P. Quayle, M.G. Briscoe, R. Howe, B.S. Rao, J. Growcott, B.R. Holloway, Br. J. Pharmacol., 112, 213P (1994).
94. J.S. Pears, M.D. Tuersley, K. Carroll, W. Webb, R.J. Ruane, D.S. Millson, A.J. Williams, Br. J. Pharmacol. 38, 161P (1994).
95. J.D. Bloom, M.D. Dutia, B.D. Johnson, A. Wissner, M.G. Burns, E.E. Largis, J.A. Dolan, T.H.Claus, J. Med. Chem., 35, 3081 (1992).
96. J.D. Bloom, T.H.Claus, Drugs Fut., 19, 23 (1994).
97. J.A. Dolan, H.A. Muenkel, M.G. Burns, S. M. Pelligrino, C.M. Fraser, F. Pietri, A.D. Strosberg, E.E. Largis, M.D. Dutia, J.D. Bloom, A.S. Bass, T.K. Tanikella, A. Cobuzzi, F.M. Lai, T.H. Claus, J. Pharmacol. Exp. Ther. 269, 1000 (1994).
98. E.E. Largis, M.G. Burns, H.A. Muenkel, J.A. Dolan, T.H. Claus, Drug Develop. Res., 32, 69 (1994).
99. T. Yoshida, N. Sakane, Y. Wakabayashi, T. Umekawa, M. Kondo, Life Sci., 54, 491 (1994).
100. T. Yoshida, N. Sakane, Y. Wakabayashi, T. Umekawa, M. Kondo, Eur. J. Endocrin. 131, 97 (1994).
101. J. Himms-Hagen, J. Cui, E. Danforth, Jr., D.J. Taatjes, S.S. Lang, B.L. Waters, T.H. Claus, Am. J. Physiol. 35, R1371 (1994).
102. J. Himms-Hagen, M. Ghorbani, T.H. Claus, Obesity Res., 1 (Suppl. 2), 90S (1993).
103. M. Ghorbani, J. Himms-Hagen, D.J. Taatjes, T.H. Claus, Int. J. Obesity 18 (Suppl. 2), 130 (1994).
104. R. Howe, B.S. Rao, B.R. Holloway, D. Stribling, J. Med Chem., 35, 1751 (1992).
105. B.R. Holloway, R. Howe, B.S. Rao, D. Stribling in "Obesity in Europe 88. Proceedings of the 1st European Congress on Obesity," P. Bjorntorp S. Rossner Eds. John Libbey, London, 1989, p. 323.
106. R. Howe, B.S. Rao, B.R. Holloway, D. Stribling, J. Med Chem., 35, 1759 (1992).
107. B.R. Holloway, R. Howe, B.S. Rao, D. Stribling, Eur. Pat. Applic. 0254532, (Filed 1986).
108. B.R. Holloway, R. Howe, B.S. Rao, D. Stribling, Amer. J. Clin. Nutr, 55, 262S (1992).
109. B.R. Holloway, R. Howe, B.S. Rao, D. Stribling, R.M. Mayers, M.G. Briscoe, J.M. Jackson, Brit. J. Pharmacol., 104, 97 (1991).
110. S. Toubro, A. Astrup, M. Hardman, Int. J. Obesity, 17(Suppl. 2), 73 (1993).
111. G.R. Goldberg, A.M. Prentice, W. Haines, P.R. Murgatroyd, Int. J. Obesity, 16, 151 (1992).
112. S.P. Quayle, R.M. Mayers, T.L. Grant, B. Holloway, Brit. J. Pharmacol, 108, 169P (1993).
113. L.J. Beeley, B.C.C. Cantello, C. Cantello, B. Christian, WO 9310074 (1993).
114. L.J. Beeley, J.M. Berge, R.L Jarvest, WO 9402493 (1994).
115. M.H. Fisher, R.J. Mathvink, O.H. Ok, E.R. Parmee, A.E. Weber WO 9418161 (1994).
116. P.Sher, US Patent 5,321,036 (1994)
117. B.R. Holloway, R.Howe, D.J. Lecount, Eur. Patent EP 056,5317 A (1993).
118. Y. Shiokawa, M. Nagano, K. Taniguchi, K. Take, T. Kato K. Tsubaki, WO 9315041 (1993).
119. R.L. Dow, S.W. Wright WO 9429290-A1 (1994).
120. A. Guidice, T. Croci, A. Bianchetti, L. Manara, Life Sci., 44,1411 (1989).
121. M. MacLachlan, A.A. Connacher, R.T. Jung, Int. J. Obesity, 15, 27 (1991).
122. A.K. Bahl, Eur. Patent EP 0556880 A (1993).

Chapter 21. The T Cell - Antigen Presenting Cell Interaction as a Site for Immunosuppressive Interventions

Zoltan A. Nagy and Damir Vidovic'
Department of Inflammation/Autoimmune Diseases
Hoffmann-La Roche Inc., Nutley, NJ 07110-1199

Introduction - The activation of T lymphocytes by antigen displayed on the surface of specialized cells, termed antigen presenting cells (APC), is the initial step of all immune responses. The activation step involves a series of molecular interactions between T cell and APC, as shown in Fig.1. Only one interaction is needed for antigen recognition; all others represent an elaborate system of cell-cell adhesion and signal amplification. In principle, disrupting any one of these interactions may lead to immunosuppression. Here, we will attempt to summarize the possible approaches aimed at interfering with the T cell-APC interaction. Subsequent steps and consequences of T cell activation, for example, intracellular signaling and the activation of effector mechanisms (lymphokines) also represent areas of pharmacological interest, but they are beyond the scope of this review. The primary therapeutic targets of immunosuppression are autoimmune diseases (AID) and transplantation. Immunosuppression should, however, be regarded as a double-edged sword: it is beneficial as long as it is targeted to a pathological response, but potentially dangerous if it compromises host defense against pathogens. This aspect will be discussed for each approach.

MOLECULES INVOLVED IN ANTIGEN RECOGNITION BY T CELLS

The Antigen Receptor of T cells(TCR) - The TCR is a disulfide-linked heterodimeric cell membrane glycoprotein composed of α and ß chains, each with a molecular weight of approximately 45 kD. Each chain comprises a variable (V), joining (J) and constant (C) region. In addition, the ß chain has a short diversity (D) region between V and J. Each region is encoded by a separate gene. The human TCR gene complex contains 13 *Vα* families each with 3 to 20 members, 50 *Jα*, 1 *Cα*, 18 *Vß*, 2 *Dß*, 14 *Jß*, and 2 *Cß* genes. During ontogeny, a single member of each α and ß gene segment-group is joined by DNA rearrangement to form a continuous *VJα* and *VDJß* gene that will be transcribed. The rearrangement is random, involving different *V(D)J* segments in each T cell. Thus, the combinatorial variation allows the generation, in principle, of an astronomical number of different receptors, distributed clonally, *i.e.*, each cell expressing only one. Additional variability is generated by random additions or deletions at the joining points, and by using the D segments in all three reading frames. Most T cells (>95%) express αß TCR, but a minority of them uses another receptor γδ, that is similar to αß. The γδ subset of T cells may serve a specialized function that is yet not fully elucidated. The three dimensional structure of TCRs is unknown. The genetic organization and function of TCR have been reviewed (1,2).

Proteins, such as monoclonal antibodies (mAbs) that bind to TCR, and prevent its interaction with APC can interfere with T cell activation. The mAbs, depending on their specificity, can be pan-TCR reactive, chain-specific, recognizing all or most α or ß chains, V region-specific, or clonotypic, specific for a particular VαVß combination. Many of these antibodies are available. In fact, the complete set of anti-V mAbs will soon be commercialized for diagnostic purposes (3). The use of mAbs with broad reactivity can be considered in transplant rejection, where a large number and variety of T cells is involved, although they have also been reported to be efficient in different

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

autoimmune disease models (4-6). The more selective mAbs can be applied in AID, in which a preferential use of a limited set of receptors has been shown. Evidence for a limited TCR usage comes from autoimmune models, but similar findings in human diseases, particularly in multiple sclerosis (MS), are also accumulating (7-9). The attraction of the approach is that mAbs specific, for example, for a single V region, would allow an immunosuppressive therapy of high selectivity, affecting only 1 to 10% of T cells (depending of the expression-frequency of the particular V region).

There is evidence that short synthetic peptides (15-20mers) corresponding to a TCR V region or joining region sequence can cause functional inactivation of T cells expressing this sequence. Thus, if T cells involved in autoimmune pathology preferentially express a particular TCR sequence, vaccination with the corresponding synthetic peptide may have a therapeutic effect (10,11). The mechanism whereby such peptides cause immunosuppression remains to be elucidated. The finding that certain TCR sequences are subject to self tolerance, and thus, cannot be used for vaccination, places specific limitations on the approach (12).

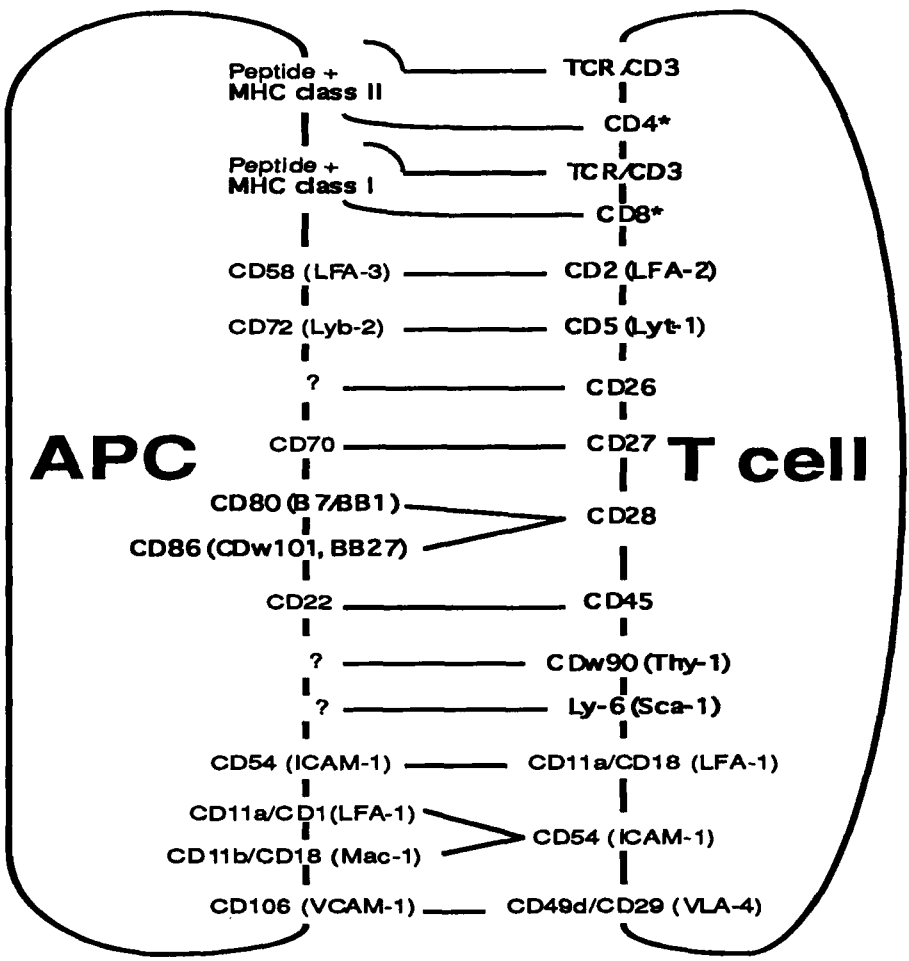


Figure 1. Molecular interactions between T cell and antigen presenting cell (APC). Molecules with known signaling function are in bold face. *Note that CD4 and CD8, although depicted on a single T cell for simplicity, are expressed on two different subsets of T cells, T-helper cells and cytotoxic T-cells, respectively.

CD3 - A complex of four polypeptides physically associated with the TCR in the cell membrane, and involved in T cell signaling via TCR (13). The polypeptides are termed γ, δ, ε and ζ, occurring either as disulfide bonded homodimers (ε-ε, ζ-ζ), or heterodimers (γ-ε, δ-ε). Their amino acid sequences are known, but the three dimensional structures have not been reported. Certain anti-CD3 mAb (*e.g.*, OKT3) cause total T cell immunosuppression, and are used successfully in clinical transplantation. The first attempt toward a small molecular weight antagonist design is the demonstration that the phosphorylation of a tyrosine-based activation motif in the cytoplasmic tail of ζ chains can be inhibited by a protein tyrosine phosphatase-resistant peptide analog (14). This analog inhibited the association of the ZAP-70 kinase with the ζ chain, thereby causing inhibition of T cell activation via the TCR-CD3 complex.

Major Histocompatibility Complex (MHC) Molecules - MHC molecules exist in two forms, class I and class II, both encoded within a single gene complex. Both class I and class II MHC genes are highly polymorphic: most loci have up to ~100 alleles in the population (15).

Class I MHC molecules are 45 kD glycoproteins integrated into the cell membrane by a hydrophobic transmembrane sequence, and also possess a short intracytoplasmic tail. They are noncovalently associated with another glycoprotein, the 12 kD beta-2 microglobulin that is not inserted into the membrane and is encoded outside the MHC. Human class I molecules have three different isotypes, termed HLA-A, -B, and -C, encoded in separate loci. The tissue expression of class I molecules is ubiquitous. The three dimensional structures of several human and murine class I molecules have been resolved (16-19). Their first and second N-terminal domains fold into a characteristic binding site consisting of a ß-pleated sheet platform flanked by two parallel α-helical portions. This binding groove contains antigenic peptides presented to the TCR for recognition. In addition, there is a binding site on the third class I domain that interacts with CD8 molecules (see below), expressed selectively on the cytotoxic subset of T cells (Tc). A critical step in Tc cell activation appears to be the simultaneous interaction of TCR and CD8 with the same class I molecule. Antigens are presented in the form of short peptides of fixed length (7 to 9 residues long) bound in an extended conformation (18,19). Most of these peptides arise from proteins synthesized inside the presenting cell. They bind to the class I groove by side chain interactions with six pockets within the groove, as well as by interactions between the peptide main chain and side chains of the binding site (17,19). The three isotypes, as well as allelic forms within a single isotype exhibit different peptide binding specificities, depending on polymorphic residues in the binding pockets that determine the peptide side chains accepted by the pockets (20,21).

Class II MHC molecules are noncovalently associated heterodimers of two glycoproteins, the 35 kD α chain and the 28 kD ß chain. Both chains are inserted into the cell membrane. A third, nonpolymorphic, non-MHC encoded 31 kD chain, termed invariant (Ii) chain, is associated with the heterodimer during biosynthesis and intracellular transport (22). The Ii chain dissociates from the heterodimer in an acidic intracellular compartment, prior to cell surface expression. In humans, class II molecules occur in three different isotypes, termed HLA-DR, -DP, and -DQ. Polymorphism in DR is restricted to the ß chain, whereas both chains are polymorphic in the DP and DQ isotypes. Class II molecules exhibit a restricted tissue distribution: they are expressed constitutively only on B lymphocytes and "professional" APC, such as dendritic cells, and their expression is inducible on cells of the monocytic lineage. The three dimensional structure of a human class II molecule, HLA-DR1 has been determined (23-25). Overall, the structure is very similar to that of class I molecules. The peptide binding site is composed of the first domains of α and ß chain, and consists of a ß-pleated sheet floor and two α-helical regions. The binding groove is open on both ends, which has important functional consequences. An additional

binding site on the second domain of ß chains interacts with the CD4 molecule, expressed selectively on helper T (Th) cells (see below). This molecule has a co-receptor function for Th cells, analogous to that of CD8 for Tc cells. Class II molecules present peptide antigens, whose length is variable (12-24 amino acids), since the binding site is open at both ends. The presented peptides arise by cleavage of endocytosed external proteins in an acidic endosomal compartment (26). However, internal proteins passing through the endocytic compartment can also be proteolytically processed and presented in a class II-bound form (27). In DR molecules, there is one major pocket in the binding site that is important for interaction with a hydrophobic anchor residue of the peptide (24). There are additional interactions with polymorphic residues that confer a degree of allotype-specificity to peptide binding (28-31). The peptide main chain also forms important hydrogen bonds with the side chains of certain conserved residues in the groove, leading to increased binding affinity (24).

Of the two classes of MHC molecules, class II is the primary target for immunosuppressive intervention for the following reasons: First, class II molecules activate Th cells that are central to immunoregulation, whereas the class I-activated Tc cells perform effector functions only. Second, most autoimmune diseases are genetically associated with certain class II alleles, and only a few with class I (32). Third, class II molecules are only expressed on cells of the immune system, whereas class I molecules are present on most somatic cells. Class II-targeted immunosuppressive approaches are aimed to interrupt the interaction of TCR of Th cells with the class II molecule plus the antigenic peptide presented, the interaction between class II and CD4 molecules (see below), the interaction between class II molecules and antigenic peptides, and the cell surface expression of class II molecules.

Protein-based class II MHC antagonist candidates include mAbs specific for class II molecules. Many, although not all anti-class II mAbs can inhibit Th cell activation, and are also efficient immunosuppressants in different animal models of transplantation and autoimmunity (33-35). The mechanism of inhibition is steric hindrance of TCR-class II interaction, although additional mechanisms, such as, depletion of class II positive cells, may also play a role (36). Certain anti-class II mAbs have been found to be toxic in primates, for unknown reasons (37,38). This finding has slowed down the development of mAb-based class II antagonists for clinical purposes.

The selectivity of immunosuppression depends on the specificity of mAb used. Some of these mAbs can recognize all class II molecules, and thus would affect all Th cells, comprising ~ 2/3 of the T cell pool. There are isotype-specific mAbs that would affect Th cells recognizing antigen presented selectively by one of the three isotypes. The proportion of T cells affected is ~10-50% depending on the expression of the particular isotype. Finally, certain mAbs recognize only one or a few allotypes within an isotype. In principle, such antibodies should only affect 10-15% of T cells. An alternative to antibody therapy is to vaccinate individuals with class II-derived synthetic peptides that can induce autoantibodies blocking self class II molecules (39). The disadvantage of this approach is that the dosing cannot be controlled. The inhibition of Th cell activation will deprive B cells of T cell help, and thus, B cell activation and antibody production will also be affected indirectly. Furthermore, anti-class II mAbs also bind to B cells directly, and this fact could lead to side effects. The therapeutic target of mAbs with broad specificity will be transplantation and graft versus host disease, whereas the more selective mAbs may be applied in autoimmune diseases with a known class II association.

Small molecular weight class II antagonists approaches are based on the discovery of interactions between the class II binding site and antigenic peptides (23-25,28-31). Only certain defined peptide residues are required for the interaction with the class II

binding cleft, whereas the side chains of other residues pointing out of the cleft and recognized by TCR are interchangeable. Thus, tightly binding ligands with altered TCR contact sites, or without such sites, can be used as potent antagonists. The principle has been proven for *in vivo*, in altered T cell responses as well as in autoimmune models (40-42). However, a peptide-based development program failed due to unfavorable pharmacokinetics of the peptides used (43). More recent approaches are aimed at the design of class II-binding non-peptidic ligands (peptidomimetics). At present, detailed structure activity relationship (SAR) information is only available for the DR isotype (28-31). DR antagonists can be designed to be either broadly reactive or allotype- selective, thus affecting ~10 to 50% of T cells. The primary targets of the antagonists are class II-associated autoimmune diseases, primarily rheumatoid arthritis (RA), multiple sclerosis (MS), and insulin-dependent, or type I diabetes mellitus (IDDM). The rationale is that the genetic association of autoimmune diseases with certain class II alleles reflects selective presentation of the disease-inducing autoantigens by the allotypes conferring disease susceptibility. Although these autoantigens are not yet defined, there is recent evidence for a correlation between peptide-binding specificity and disease association in the case of RA (44).

Regulation of class II gene expression may provide an alternative target to achieve a similar effect to that of mAbs and small antagonist molecules discussed above. Recent research in this area has identified molecules that regulate class II gene transcription, although it is not yet clear how specific this regulation is (45).

Although class I molecules are usually not considered as targets for immunosuppression for reasons discussed above, recent evidence suggests that they are involved in T cell signaling, and may therefore become interesting targets (46).

The Ligand of TCR - As discussed above, the TCR recognizes antigens in the form of short peptides presented by MHC molecules. Although the molecular details of recognition are unresolved, it appears that the TCR interacts with solvent exposed side chains of the bound peptide as well as with certain residues of the MHC peptide-binding site itself (47). Thus, the ligand is peptide+MHC. Interestingly, the affinity of TCR-ligand interaction is very low, K_d ~10^{-5}-10^{-7}M, and may explain the necessity for additional molecular interactions that stabilize TCR-ligand (or T cell-APC) contact, and amplify TCR-derived signals (48-50).

Tolerance induction by the ligand represents a possible way to achieve selective immunosuppression. Its rationale is that the recognition of a ligand alone, in the absence of co-stimulatory signals provided by the APC, leads to antigen-specific inactivation or anergy of T cells (51). Thus, were the autoantigenic ligands precisely defined, a selective silencing of T cells responsible for autoimmune pathology could be achieved (52). Experimental tolerance has been induced by injection of the antigenic peptide in a soluble form, *i.e.*, without adjuvants, intravenously; by injecting soluble MHC molecules complexed with the antigenic peptide; and by administering the antigen through mucous membranes, such as orally or by inhalation (53-56). The therapeutic utility of at least the first two application modes requires precise identification of the relevant autoantigenic peptides. Tolerance induction is the most selective way of immunosuppression, since it only inactivates the set of T cell clones recognizing a single peptide. Although the number of clones used for the recognition of a peptide may be variable, the proportion of T cells affected must be below the 1% level.

TCR antagonism is a recently discovered phenomenon with a potential therapeutic utility. Apparently, peptides that do not perfectly "fit" to the TCR, yet bear resemblance to the "fitting" ligand, can induce an inactivating signal in the T cell even in the presence of the necessary co-stimulatory signals (57). TCR antagonist peptides have to be presented in an MHC-bound form similar to the ligand or bound MHC antagonists.

The design of antagonists requires knowledge of the ligand, and thus, the therapeutic utility of the phenomenon is limited by the same factors as that of tolerance induction. The selectivity of TCR antagonism should be in the same range as that of tolerance induction (58).

ACCESSORY MOLECULES

In the process of MHC/peptide recognition, T cells engage in a series of molecular interactions with APC in order to facilitate cell-cell adherence, and to receive co-stimulatory signals. These functions are mediated by accessory molecules, that can be subdivided into 3 families: (1) co-receptors for MHC recognition (CD4 and CD8); (2) cell adhesion molecules (integrins: LFA-1, Mac-1, VLA-4); and (3) co-stimulatory molecules (CD2, CD5, CD26, CD27, CD28, CD45, CDw90, and Ly-6). Each adhesion and co-stimulatory molecule has a cellular ligand on the APC (see in Fig.1). The amino acid sequences of molecules have been determined, but the structures for most of them (except CD2, CD4, CD8, CD54, and CD106) are unknown.

Co-Receptors for MHC: CD4 and CD8 - TCRs recognize peptides bound to either class I or class II MHC molecules. Simultaneously, CD4 molecules expressed exclusively on Th cells contact a conserved site on the peptide-presenting class II molecules, and CD8 molecules expressed only on Tc cells engage a site on the antigen-presenting class I molecules (59). Thus, T cells ligate the antigen presenting MHC molecule by two different receptors, thereby strengthening the interaction, and allowing for distinct Th cell-class II and Tc cell-class I pairing. The co-receptors may also contribute to intracellular signaling following antigen recognition. CD4 is a single polypeptide with four immunoglobulin (Ig)-like extracellular domains. CD8 is a disulfide-bonded dimer of either two α, or an α and a β chain. Each chain has an N-terminal Ig-like domain. The crystal structure of the first two Ig-like domains of CD4, and the N-terminal Ig-like domain of CD8 α have been determined (60-62). The three-dimensional structure of these domains is almost identical to Ig variable (V) domains, and their dimerization in CD8 is also similar to that of Ig heavy and light chain V domains. However, the two V-like domains of CD4 are arranged differently to form a rod shaped molecule. Thus, despite similarities, the two co-receptors will use partially different surfaces for contacting MHC molecules. The contact sites on the MHC molecules have been mapped to the α3 domain of class I, and the β2 domain of class II molecules (63-66). A site partially overlapping with the class II binding site on the N-terminal domain of CD4 interacts with the gp120 glycoprotein of HIV virus (67), and is used by the virus as a receptor for entering Th cells.

Anti-CD4 mAbs have been used extensively for the treatment of experimental autoimmune diseases in animal models (68). However, in human trials the mAbs caused long-lasting total depletion of $CD4^+$ cells by the induction of apoptotic cell death upon ligation (69). Anti-CD4 mAb therapy has been shown to induce tolerance to antigens encountered during the treatment (70). Thus, self tolerance can be re-established or long-lasting transplantation tolerance induced by mAb treatment (71). Another approach to anti-CD4 antibody therapy is to generate autoantibodies by active immunization with synthetic peptides of CD4 (72). The limitations of this approach have been discussed in the section on MHC. The first example of a small CD4 antagonist molecule is a cyclic peptide analog of the CDR3 region of CD4 that inhibits experimental autoimmune encaphalomyelitis (EAE), an animal model of MS (73).

The CD8 molecule is usually not considered as a pharmacological target, since it is expressed selectively on Tc cells performing effector but not regulatory functions. Nevertheless, the blocking of CD8-class I interaction results in inhibition of T cell-mediated killing, and thus, the principle can be used for therapeutic purposes. One target may be IDDM, where $CD8^+$ Tc cells appear to cause the autoimmune

damage to pancreatic beta cells (74). However, all therapeutic approaches targeting these two co-receptors are of low selectivity. The blocking of CD4 affects 2/3, and that of CD8 1/3 of the total T cell pool.

Integrins - Integrins represent a family of cell surface proteins mediating cell adhesion functions. This review is restricted to integrins relevant to T cell-APC interaction. More detailed reviews are available (75-76).

LFA-1, Mac-1 - LFA-1 is a heterodimer composed of 180 kD α chain (CD11a) and a 95 kD ß chain (CD18). It is expressed on virtually all leukocytes. Its ligand is CD54 (ICAM-1), a 90 kD cell surface protein, with a broad cellular expression. The crystal structure of the N-terminal domain of human CD54 has been reported (77). Since LFA-1 and CD54 are expressed on both T cells and APC, their ligation occurs in both directions, thus strengthening T cell-APC adhesion. The expression of both molecules can be induced, *in vivo*, at inflammation sites (78,79). Mac-1 is another ligand to CD54 that is expressed on monocytes only. It is structuraly similar to LFA-1, consisting of CD18 and a 150 kD α chain, termed CD11b. Certain anti-LFA-1 and anti-CD54 mAbs were found very efficient in treating experimental autoimmune uveoretinitis in mice, and autoimmune glomerulonephritis in rat (78,79). Furthermore, anti-LFA-1 mAbs could both prevent and cure experimental autoimmune neuritis in rat, and prevent experimental autoimmune thyroiditis in mice (80-82). It has been reported that co-ligation of TCR and LFA-1 induces activation-dependent cell death, a mechanism that may be exploited in immunosuppression (83).

VLA-4 - VLA-4 is a heterodimer composed of a 150 kD α (CD49d) and 130 kD ß (CD29) chain. It is widely expressed on virtually all leukocytes. It binds to a matrix protein, fibronectin, as well as to its cellular ligand CD106 (VCAM-1; 100 kD). The crystal structure of the first two domains of CD106 comprising the VLA-4 binding site has recently been determined (84,85). CD106 is expressed by cytokine activated APC, thus stabilizing the adhesion of T cells to the former. Certain VLA-4 specific mAbs can prevent the onset of type I diabetes in NOD mice (86,87). Thus, this molecule is a potential target for drugs to be applied in autoimmune disease. Recently, a cyclic octapeptide has been shown to act as a potent small molecular weight antagonist to VLA-4 mediated cell adhesion, *in vitro* (88).

Table 1. Co-stimulatory Molecules, Their Expression and Ligands

Co-stimulatory Molecule	Cellular Expression	Known ligand(s)	Refs.
CD2 (LFA-2)	T-cells	CD58 (LFA-3)	89-92
CD5 (Lyt-1)	T- and some B-cells	CD72 (Lyb-2)	93
CD26 (DPP-IV)	activated T-cells, B- cells, macrophages		94,95
CD27	activated T- and B-cells, plasma-cells	CD70	96,97
CD28	T-, plasma-, and activated B-cells	CD80 (B7, BB1) CD86 (CDw101, BB27, BA27)	98-101
CD45 (LCA, T200)	leukocytes	CD22 (BL-CAM, Lyb-8)	102-105
CDw90 (Thy-1)	T-cells, neurons		106,107
Ly-6 (Sca-1)	T-, B-cells, monocytes, hematopoietic progenitors		108-110

Co-stimulatory Molecules - In addition to TCR/CD3 signaling, T cells require secondary signals in order to become activated. These signals are provided by numerous co-stimulatory glycoproteins after their binding to the corresponding ligands on APC.

Potential targets for blocking T-cell / APC interactions are listed in Table 1. The selectivity of immunosuppressive therapies targeting cell-adhesion and co-stimulatory molecules will depend on the cellular expression of the targeted molecule. Clearly, the expression of most of these molecules is not restricted to T cells or APC, in most instances not even to lymphoid cells. Thus, in general, a low degree of selectivity is expected. Tolerance induction by CD28 antagonism may represent an exception, since it will have the high selectivity of tolerance induction.

Conclusion - The T cell-APC interaction provides a large number of molecular targets for immunosuppressive pharmacological intervention. Antibody inhibition data suggest that interrupting any of the molecular interactions between these two cells can potentially result in immunosuppression. Molecules directly involved in antigen recognition, *i.e.*, TCR and MHC molecules have the greatest potential as targets for immunosuppression because of their high selectivity. Accessory molecules, although promising targets, allow a lower degree of selectivity. Small antagonist molecule approaches to these targets are relatively scarce, and structure-based approaches for the few molecules of known structure (*e.g.*, MHC, CD4, VLA-4) are in their infancy. Therefore, these molecules represent largely unexploited targets for medicinal chemistry.

References

1. R.K. Wilson, E. Lai, P. Concannon, R.K. Barth, L.E. Hood, Immunol. Rev., 101, 149 (1988).
2. M.B. Brenner, J.L. Strominger, M.S. Krangel, Adv. Immunol., 11, 340 (1990).
3. D.N. Posnett, R. Sinhga, S. Kabak, C. Russo, J. Exp. Med., 179, 609 (1994).
4. P. Sempe, P. Bedossa, M.-F. Richard, M.-C. Villa, J.-F. Bach, C. Boitard, Eur. J. Immunol., 21, 1163 (1991).
5. T.J. Goldschmidt, R. Holmdahl, Eur. J, Immunol., 21, 1327 (1991).
6. S. Yoshino, L.G. Cleland, G. Mayrhofer, R.R. Brown, J.H. Schwab, J. Immunol., 146, 4187 (1991).
7. H. Acha-Orbea, D.J. Mitchell, L. Timmermann, D.C. Wraith, G.S. Tausch, M.K. Waldor, S.S. Zamvil, H.O. McDevitt, L. Steinman, Cell, 54, 263 (1989).
8. J.R. Oksenberg, S. Stuart, A.B. Begovich, R.B. Bell, H.A. Erlich, L. Steinman, C.C.A. Bernard, Nature ,345, 344 (1990).
9. B.L. Kotzin, S. Karuturi, Y.K. Chou, J. Lafferty, J.M. Forrester, M. Better, G.E. Nedwin, H. Offner, A.A. Vandenbark, Proc. Natl. Acad. Sci. USA, 88, 9161 (1991).
10. M.D. Howell, S.T. Winters, T. Olee, H.C. Powell, D.J. Carlo, S.W. Borstoff, Science, 246, 668 (1989).
11. H. Offner, G.A. Hashim, A.A. Vandenbark, Science 251, 430, (1991).
12. F. Falcioni, D. Vidovic', E.S. Ward, D. Bolin, G. Singh, H. Shah, B. Ober, Z.A. Nagy, J. Exp. Med., in press (1995)
13. H. Clevers, B. Alarcon, T. Wileman, C. Terhorst, Ann. Rev. Immunol., 6, 629 (1988).
14. R.L. Wange, N. Isakov, T.R. Burke, A. Otaka, P.P. Roller, J.D. Watts, R. Aebersold, L.E. Samelson, J. Biol. Chem., 270, 944 (1995).
15. T.H. Hansen, B.M. Carreno, D.H. Sachs, in "Fundamental Immunology" W.E. Paul, Ed., Raven Press, New York, N.Y., 1993, p. 577.
16. P.J. Bjorkman, M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, D.C. Wiley, Nature, 329, 506 (1987).
17. T.P.J. Garrett, M.A. Saper, P.J. Bjorkman, J.L. Strominger, D.C. Wiley, Nature, 342, 692 (1989).
18. D.R. Madden, J.C. Gorga, J.L. Strominger, D.C. Wiley, Nature, 353, 321 (1991).
19. D.H. Fremont, M. Matsumura, E.A. Stura, R.A. Peterson, I.A. Wilson, Science, 257, 919 (1992).
20. K. Falk, O. Rötzschke, S. Stevanovic' G. Jung, H.-G. Rammensee, Nature, 351, 290 (1991).
21. O. Rötzschke, K. Falk, S. Stevanovic', G. Jung, H.-G. Rammensee, Eur. J. Immunol., 22, 277 (1992).
22. P. Cresswell, Annu. Rev. Immunol., 12, 259 (1994).
23. J.H. Brown, T.S. Jardetzky, J.C. Gorga, L.J. Stern, R.G. Urban, J.L. Strominger, D.C. Wiley, Nature, 364, 33 (1993).
24. J.C. Stern, J.H. Brown, T.S. Jardetzky, J.C. Gorga, R.G. Urban, J.L. Strominger, D.C. Wiley, Nature, 368, 215 (1994).
25. T.S. Jardetzky, J.H. Brown, J.C. Gorga, L.J. Stern, R.G. Urban, Y. Chi, C. Stauffacher, J.L. Strominger, D.C. Wiley, Nature, 368, 711 (1994).
26. R.N. Germain, Cell, 76, 287 (1994).
27. R.M. Chicz, R.G. Urban, W.S. Lane, J.C. Gorga, L.J. Stern, D.A.A. Vignali, J.L. Strominger, Nature, 358, 764 (1992).

28. J. Hammer, B. Takacs, F. Sinigaglia, J. Exp. Med., 176, 1007 (1992).
29. J. Hammer, P. Valsasini, K. Tolba, D. Bolin, J. Higelin, B. Takacs, F. Sinigaglia, Cell, 74, 197 (1993).
30. J. Hammer, C. Belunis, D. Bolin, J. Papadopoulos, R.Walsky, J. Higein, W. Danho, F. Sinigaglia, Z.A. Nagy, Proc. Natl. Acad. Sci. USA, 91, 4456 (1994)
31. J. Hammer, E. Bono, F. Gallazzi, C. Belunis, Z.A. Nagy, F. Sinigaglia, J. Exp. Med., 180, 2353 (1994).
32. G.T. Nepom, H. Erlich, Annu. Rev. Immunol. 9, 493 (1991).
33. L. Steinman, J.T. Rosenbaum, S. Sriram, H.O. McDevitt, Proc. Natl. Acad. Sci. USA, 78, 7111 (1981).
34. M. Jonker, R. van Lambalgen, D.J. Mitchell, S.K. Durham, L. Steinman, J. Autoimmun., 1, 399 (1988).
35. R.M. Smith, A. Morgan, D.C. Wraith, Immunol., 83, 1 (1994).
36. J.-P. Truman, M.L. Ericson, C.J.M. Choqueux-Seebold, D.J. Charron, N.A. Mooney, Internatl. Immunol., 6, 887 (1994).
37. R. Billing, S. Chatterjee, Translpant. Proc., 15, 649 (1983).
38. M. Jonker, F.J.M. Nooij, G. den Butter, R. van Lambalgen, A.J. Fuccello, Translantation, 45, 677 (1988).
39. D.J. Topham, B. Nag, S. Arimilli S. Sriram, Proc. Natl. Acad. Sci. USA, 91, 8005 (1994).
40. L. Adorini, S. Muller, F. Cardinaux, P.V. Lehmann, F. Falcioni, Z.A. Nagy, Nature, 334, 623 (1988)
41. D.C. Wraith, D.E. Smilek, D.J. Mitchell, L. Steinman, H.O. McDevitt, Cell, 59, 247 (1989).
42. U. Hurtenbach, E. Lier, L. Adorini, Z.A. Nagy, J. Exp. Med., 177, 1499 (1993).
43. G.Y. Ishioka, L. Adorini, J.-C. Guery, F.C.A. Gaeta, R. LaFond, J. Alexander, M.F. Powell, A. Sette, H.M. Grey, J. Immunol., 152, 4310 (1994).
44. J. Hammer, F. Gallazzi, E. Bono, R.W. Karr, J. Guenot, P. Valsasini, Z.A. Nagy, F. Sinigaglia, J. Exp. Med., 181, in press (1995).
45. V. Steimle, C.-A. Siegrist, A. Mottet, B. Lisowska-Grospierre, B. Mach, Science, 265, 107 (1994).
46. D.M. Smith, J.A. Bluestone, D.R. Jeyarajah, M.H. Newberg, V.H. Engelhard, J.R. Thistlethwaite, Jr., E.S. Woodle, J. Immunol., 153, 1054 (1994).
47. M.M. Davis, P.J. Bjorkman, Nature, 334, 395 (1988).
48. K. Matsui, J.J. Boniface, P.A. Reay, H. Schild, B. Fazekas de St. Groth, M.M. Davis, Science, 254, 1788 (1991).
49. S. Weber, A. Traunecker, F. Oliveri, W. Gerhard, K. Karjalainen, Nature, 356, 793 (1992).
50. M. Corr, A.E. Slanetz, L.F. Boyd, M.T. Jelonek, S. Khilko, B. Al-Ramadi, Y.S. Kim, S.E. Maher, A.L.M. Bothwell, D.H. Margulies, Science, 265, 946 (1994).
51. P. Matzinger, Annu. Rev. Immunol., 12, 991 (1994).
52. A. Gaur, B. Wiers, A. Liu, J. Rothbard, C.G. Fathman, Science, 258, 1491 (1992).
53. E.R. Kearney, K.A. Pape, D.Y. Loh, M.K. Jenkins, Immunity, 1, 327 (1994)
54. S.D. Sharma, B. Nag, X.-M. Su, D. Green, E. Spack, B.R. Clark, S. Sriram, Proc. Natl. Acad. Sci. USA, 88, 11465 (1991).
55. A. Miller, O. Lider, H.L. Weiner, J. Exp. Med., 174, 791 (1991).
56. B. Metzler, D.C. Wraith, Internatl. Immunol., 5, 1159 (1993).
57. J. adrenas, R.L. Wange, J.L. Wang, N. Isakov, L.E. Samelson, R.N. Germain, Science, 267, 515 (1995).
58. V.K. Kuchroo, J.M. Greer, D. Kaul, G. Ishioka, A. Franco, A. Sette, R.A. Sobel, M.B. Lees, J. Immunol., 153, 3326 (1994).
59. B.E. Bierer, B.P. Sleckman, S.E. Ratnofsky, S.J. Burakoff, Ann. Rev. Immunol., 7, 579 (1989).
60. J. Wang,Y.W. Yan, T.P. Garrett, J.H. Liu, D.W. Rodgers, R.L. Garlick, G.E. Tarr, Y. Husain, E.L. Reinherz, S.C. Harrison, Nature, 348, 411 (1990).
61. S.-E. Ryu, P.D. Kwong, A. Truneh, T.G. Porter, J. Arthos, M. Rosenberg, X.P. Dai, N.H. Xuong, R. Axel, R.W. Sweet, Nature, 348, 419 (1990).
62. R. Leahy, R. Axel, W.A. Hendericksón, Cell, 68, 1145 (1992).
63. T.A. Potter, T.V. Rajan, R.F. Dick, J.A. Bluestone, Nature, 337, 73 (1989).
64. R.D. Salter,R.J. Benjamin, P.K. Wesley, S.E. Buxton, T.P. Garrett, C. Clayberger, A.M. Krensky, A.M. Norment, D.R. Littman, P. Parham, Nature, 345, 41 (1990).
65. R. König, L.-Y. Huang, R.N. Germain, Nature, 356, 796 (1992).
66. G. Cammarota, A. Scheirle, B. Takacs, D.M. Doran, R. Knorr, W. Bannwrth, J. Guardiola, F. Sinigaglia, Nature, 356, 799 (1992).
67. L.K. Clayton, M. Sieh, D.P. Pious, E.L. Reinherz, Nature, 339, 548 (1989).
68. S. Sriram, G.E. Ranges, Concepts Immunopathol. 4, 275 (1987).
69. E.H. Choy, J. Adjaye, L. Forrest, G.h. Kingsley, G.S. Panayi, Eur. J. Immunol., 23, 2676 (1993).
70. S.P. Cobbold, G. Martin, S. Qin, H. Waldman, Nature, 323, 164 (1986).
71. P. Hutchings, N. Parish, L. O'Reilly, K. Dawe, I.M. Roitt, A. Cooke, Autoimmunity, 15, 21 (1993).
72. P. Lanza, R. Billetta, S. Antonenko, M. Zanetti, Proc. Natl. Acad. Sci. USA, 90, 11683 (1993)
73. B.A. Jameson, J.M. McDonnell, J.C. Marini, R. Korngold, Nature ,368, 744 (1994).

74. C. Thivolet, A. Bendelac, P. Bedossa, J.-F. Bach, C. Carnaud, J. Immunol., 146, 85 (1991).
75. R.S. Larson, T.A. Springer, Immunol. Rev., 114, 181 (1990).
76. R.O. Hynes, Cell, 69, 11 (1992).
77 P.R. Kolatkar, M.A. Oliveira, M.G. Rossmann, A.H. Robbins, A.K. Katti, H. Hoover-Litty, C. Forte, J.M. Greve, A. McClelland, N.H. Olson, J. Mol. Biol., 225, 1127 (1992).
78. S.M. Whitcup, L.R. DeBarge, R.R. Caspi, R. Haming, R.B. Nussenblatt and C.C. Chan, Clin. Immunol. Immunopathol., 67, 143 (1993).
79. K. Nishikawa, Y.J. Guo, M. Miyasaka, T. Tamatani, A.B. Collins, M.S. Sy, R.T. McCluskey and G. Andres, J. Exp. Med., 177, 667 (1993)
80. J.J. Archelos, M. Maurer, S. Jung, K.V. Toyka and H.P. Hartung, Brain, 116, 1043 (1993).
81. J.J. Archelos, M. Maurer, S. Jung, M. Miyasaka, T. Tamatani, K.V. Toyka and H.P. Hartung, Lab. Invest., 70, 667 (1994).
82. R.A. Metcalfe, N. Tandon, T. Tamatani, M. Miyasaka and A.P. Weetman, Immunology, 80, 493 (1993).
83. N.K. Damle, K. Klussman, G. Leytze, A. Aruffo, P.S. Linsley, J.A. Ledbetter, J. Immunol., 151, 2368 (1993).
84. J.H. Wang, B. Pepinsly, M. Karpusas, J.H. Liu, L. Osborn, Proteins: Struct. Funct. Genet., 20, 287 (1994).
85. E.Y. Jones, K. Marion, M.J. Bottomley, R.C. Robinson, P.C. Driacoll, R.M. Edwards, J.M. Clements,T.J. Dudgeon, D.I. Stuart, Nature, 373, 539 (1995).
86. X.D. Yang, N. Karin, R. Tisch, L. Steinman and H.O. McDevitt, Proc. Natl Acad. Sci. USA, 90, 10498 (1993).
87. L.C. Burkly, A. Jakubowski and M. Hattori, Diabetes, 43, 529 (1994).
88. P.M. Cardarelli, R.R. Cobb, D.M. Nowlin, W. Scholz, F. Gorcsan, M. Moscinski, M. Yasuhara, S.-L. Chiang, T.J. Lobl, J. Biol. Chem., 269, 18668 (1994).
89. D.L. Bodian, E.Y. Jones, H. Karl, D.I. Stuart, S.J. Davis, Structure (London), 2, 755 (1994).
90. J.C. Reed, W. Tadmori, M. Kamoun, G. Koretzky, P.C. Nowell, J. Immunol.,134, 1631 (1985).
91. W. Holter, O. Fischer, H. Majdic, H. Stockinger, W. Knapp, J. Exp. Med., 163, 654 (1986).
92. V.A. Boussiotis, G.J. Freeman, J.D. Griffin, G.S. Gray, J.G. Gribben, L.M. Nadler, J. Exp. Med., 180, 1665 (1994).
93. B. Subbarao, D.E. Mosier, J. Exp. Med., 159, 1796 (1984).
94. T. Tanaka, D. Camerini, D. Seed,Y. Torimoto, N.H. Dang, J. Kameoka, H.N. Dahlberg, S.F. Schlossman, C. Morimoto, J. Immunol., 149, 481 (1992).
95. N.H. Dang, Y. Torimoto, K. Shimamura, T. Tanaka, J.F. Daley, S.F. Schlossman, C. Morimoto, J. Immunol., 147, 2825 (1991)
96. R.A.W. van Lier, J. Borst, T.M. Vroom, H. Klein, P. van Mourik, W.P. Zeijlemaker, C.J.M. Melief, J. Immunol., 139, 1589 (1987).
97. K. Agematsu, T. Kobata, K. Sugita, G.J. Freeman, M.P. Beckmann, S.F. Schlossman, C. Morimoto, J. Immunol., 153, 1421 (1994).
98. A. Aruffo, B. Seed, Proc. Natl. Acad. Sci. USA 84, 8573 (1987).
99. G.J. Freeman, A.S. Freedman, J.M. Segil, G. Lee, J.F. Whitman, L.M. Nadler, J. Immunol., 143, 2714 (1989).
100. N. Nabavi, G.J. Freeman, A. Gault, D. Godfrey, L.M. Nadler, L.H. Glimcher, Nature, 360, 266 (1992).
101. D.R. Milich, P.S. Linsley, J.L. Hughes and J.E. Jones, J. Immunol., 153, 429 (1994).
102. I.S. Trowbridge, J. Biol. Chem., 266, 23517 (1991).
103. I. Stamenkovic, D. Sgroi, A. Aruffo, M.S. Sy, T. Anderson, Cell, 66, 1133 (1991).
104. J.A. Ledbetter, N.K. Tonks, E.H. Fischer, E.A. Clark, Proc. Natl. Acad. Sci. USA, 85, 8628 (1988).
105. J.T. Pingel, M.L. Thomas, Cell, 58, 1055 (1989).
106. A.F. Williams, J. Gagnon, Science, 216, 696 (1982).
107. R. Johnson, D.W. Lancki, F.W. Fitch, J. Immunol., 151, 2986 (1993).
108. E.M. Shevach, P.E. Korty, Immunol. Today, 10, 195 (1989).
109. P.M. Flood, J.P. Dougherty, Y. Ron, J. Exp. Med., 172, 115 (1990).
110. D.H. Presky, M.G. Low, E.M. Shevach, J. Immunol., 144, 860 (1990).

Chapter 22. Chemokines as Therapeutic Targets

Kelvin Cooper and Kuldeep Neote
Pfizer Central Research, Groton, CT 06340

Introduction - The chemokines are pro-inflammatory mediators that are identified by the presence of a conserved four-cysteine motif and whose primary functions are the control of leukocyte chemotaxis and activation. Chemokines are divided into two distinct families, the C-X-C class and the C-C class, referring to the presence or absence of an amino acid between the first pair of conserved cysteine residues at the amino-terminus. Based on their biological activity, the chemokines have been postulated to play a pivotal role in the extravasation and subsequent infiltration of leukocytes into sites of inflammation and immune reactivity. This chapter will review the most recent advances and discoveries concentrating on the structure and function of the chemokines and their receptors, their *in vitro* and *in vivo* biological activities, and how modulation of their activity might present new therapeutic opportunities. The main focus will be on the known human proteins while the less typical C-X-C chemokines PF4 and IP-10 will not be discussed. Given their immune cell infiltrating properties, the chemokines have the potential to be used as therapeutic vaccines against tumors by enhancing immune responses to tumors. Several excellent reviews have been written about the chemokines (1-6).

C-X-C CHEMOKINES

The most well studied member of the C-X-C family of chemokines is IL-8, originally known as NAP-1. IL-8 expression is induced by cytokines such as IL-1, TNFα, IL-3, IL-7 and GM-CSF and by the lipid mediator LTB_4 (1, 7). It is expressed by monocytes, macrophages, neutrophils, epithelial cells, fibroblasts and some tumor cells (4). GROα, also known as melanoma growth stimulating factor (MGSA), has 43% sequence identity with IL-8 and is secreted from monocytes, neutrophils, endothelial cells, and fibroblasts (5). GROβ and GROγ are 42 and 40% identical to IL-8 respectively, and are secreted from similar cellular sources. NAP-2 has 46% identity to IL-8 and is produced by platelets. The sources of ENA-78 are lung epithelial tissue, macrophages, endothelial cells, and fibroblasts (1, 5).

In Vitro Activity - IL-8 functions primarily as a neutrophil chemoattractant and activator, inducing expression of adhesion molecules such as CD11b/CD18 ($\alpha_M\beta_2$) and CD11c/CD18 ($\alpha_X\beta_2$) on the surface of the neutrophil, stimulating shape change, promoting the release of granular enzymes such as elastase, gelatinase, and myeloperoxidase, and induction of the respiratory burst (1). IL-8 induces the release of histamine and leukotrienes from GM-CSF, IL-3 or IL-5 pretreated basophils and will induce chemotaxis of GM-CSF or IL-3 pretreated eosinophils, although less effectively than PAF or C5a. IL-8 has little effect on monocytes (even though monocytes bear IL-8 receptors) or T-cells (1). The GRO family has similar biological properties to IL-8 as chemoattractants to neutrophils in the same concentration range (0.3 to 1 nM), but induces granule exocytosis at 50% lower concentrations and induces a very weak respiratory burst response (1, 5). NAP-2 also has similar properties as the GRO family on neutrophils but with weaker effects on granule exocytosis and the respiratory burst (5). Likewise, ENA-78 has similar biological properties to IL-8 as a chemoattractant for neutrophils (8).

In Vivo Activity - The intradermal injection of IL-8 in rabbits gives rise to an extensive accumulation of neutrophils accompanied by plasma exudation (1). The recruitment is rapid and long lasting. A similar pattern is observed with intradermal injection in humans, where there is no neutrophil degranulation, no accumulation of eosinophils or

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

basophils, and no weal or flare (5). An IL-8 receptor knock out mouse has been produced and all IL-8 induced neutrophil responses *in vitro* and *in vivo* are drastically impaired (9). In addition, the knockout mouse displays lymphadenopathy, resulting from an increase in B-cells and splenomagaly resulting from an increase in metamyelocytes and neutrophils. The observed phenotype could be a consequence of impairment in neutrophil trafficking and therefore neutrophil function or, alternatively, it suggests that IL-8 plays a critical role in neutrophil and B-cell development and expansion. It also gives strong evidence of target validation by showing that an inactive IL-8 receptor will prevent neutrophil infiltration into tissues (5).

Pathophysiology - IL-8 is linked to a variety of diseases, particularly psoriasis where it has been isolated in high quantities from psoriatic lesions and is thought to be responsible for neutrophil invasion into the microabscesses of psoriatic skin (5). IL-8 can also be recovered from eczema skin, although there is no neutrophil invasion in eczema skin (10). High levels of IL-8 have also been recovered from the inflamed joints of patients suffering with rheumatoid arthritis, osteoarthritis and gout (11, 12). There is a significant correlation between disease severity and the amount of IL-8 recovered in bronchoalveolar lavage in lung diseases characterized by a high neutrophil infiltrate such as idiopathic pulmonary fibrosis and adult respiratory distress syndrome (13-15). A similar correlation has been observed in multiple organ failure patients (16). The influx of neutrophils which accompanies infection has also been connected to the presence of IL-8. For example, high concentrations of IL-8 have been isolated from the middle ear of patients with otitis media and serum samples of patients with adenovirus infection (17, 18). Colonic mucosa from patients suffering from Crohn's disease or ulcerative colitis contain significantly more IL-8 than mucosal tissue from normal colon (19). *In situ* hybridization with IL-8 anti-sense RNA probes and correlation with histological examination of resected bowel segments located the IL-8 expression to areas of inflammation and mucosal destruction (19). In reperfusion injury, an IL-8 Mab has been shown to prevent damage in a lung injury model in rabbits (20).

Recent evidence has shown that constituitive expression of GROα and GROβ is increased in osteoarthritic and normal synovial fibroblast cell lines compared to rheumatoid synovial fibroblast cell lines, suggestive of an alternative pathway of cytokine activation that may reduce the proliferative and erosive response to chronic arthritis (21). The GRO family has also been linked to skin disease and to a decrease in collagen expression by human fibroblasts (22, 23). Although the GRO family interacts at the IL-8 receptor, there is evidence for a GROα specific receptor which does not bind IL-8 (24, 25). NAP-2 is produced by the proteolysis of Platelet Basic Protein (PBP), CTAP-III and ß-TG (the latter two being products of limited proteolysis of PBP), which are released from aggregating platelets. NAP-2 induces a neutrophil infiltrate which may be involved in the reopening of the thrombotic vessel (26). ENA-78 can be recovered from the inflamed joints of RA patients and up to 42% of chemotactic activity of joint fluid can be blocked by anti ENA-78 antibodies (26).

IL-8 Receptor/ligand Interactions - Receptor binding assays predicted the presence of two distinct high affinity binding sites for IL-8 on neutrophils, one that binds IL-8 only and a second that binds IL-8, MGSA/GROα , GROβ, GROγ and NAP-2 (27, 28). Receptor desensitization and affinity cross-linking studies confirmed these observations (28, 29). Unequivocal proof for the presence of two receptors was obtained when two IL-8 receptors were identified by molecular cloning (30, 31). All cloned chemokine receptors belong to the superfamily of seven transmembrane G-protein coupled receptors (GCRs). The type A receptor binds IL-8 only, while the type B receptor binds IL-8 and other C-X-C chemokines (32). The two IL-8 receptors are ~70% identical in amino acid sequence (32). The binding specificity of the receptors is dictated by the N-terminus since switching the N-terminus of the type B

receptor onto the type A receptor confers type B receptor binding (i.e. IL-8 and MGSA) in the resulting chimeric receptor (33, 34).

Structure/function Studies - The three-dimensional structure of IL-8 has been determined and indicates that IL-8 exists as a homodimer (35-37). Site-directed mutagenesis and chemically synthesized analogs of IL-8 have identified critical residues that confer high affinity binding. The most important residues identified for high affinity binding are $E_4L_5R_6$ (38-40). The importance of the ELR motif is underscored by its absence in related C-X-C chemokines PF4 and IP10 which have weak activities towards neutrophils (41). Furthermore, substituting ELR for the natural DRL sequence in PF4 confers neutrophil binding, chemotaxis, and degranulation and enzyme release (42). However, the ELR motif is not sufficient since several linear and cyclic ELR-containing peptides are not antagonistic indicating that additional binding interactions are necessary (42, 43). Site-directed mutagenesis has been used to map functionally important amino acid residues in the type A receptor that bind to IL-8 including a putative disulfide bond between the N-terminus and the third extracellular loop (44, 45). Both the second and third extracellular loops were shown to interact with IL-8 and contribute to IL-8 binding. Mutating charged residues within these loops abolished IL-8 binding (45).

Signal transduction: Stimulation of effector cells with IL-8 leads to the respiratory burst, by activation of NADPH oxidase, degranulation and chemotaxis. These processes occur via uncoupling and activation of heterotrimeric G-proteins upon ligand binding (46). Consequently, signal transduction pathways are activated that result in mobilization of intracellular Ca^{++} stores, production of inositol triphosphate via activation of phospholipase C and PKC activation (47, 48). The end result is unresponsiveness of the receptor due to a combination of receptor phosphorylation (presumably on serine/threonine residues) and intracellular receptor uptake (49). The rise in Ca^{++} levels is essential for exocytosis and the respiratory burst, whereas chemotaxis is dependent on phospholipid turnover and phosphorylation, presumably by MAP kinases (50). Recently, reconstitution of the signal transduction events in a heterologous system by gene transfection has directly indicated that $G_{i\alpha 2}$, $G_{\beta 1}$, $G_{\gamma 2}$, together with the phospholipase C-β2 isoform, are necessary for the type A receptor to induce phospholipid turnover (51). These studies are vital to elucidating the precise mechanism of signal transduction and could potentially lead to identification of molecular targets for drug intervention.

C-C CHEMOKINES

C-C chemokines comprise at least ten cytokines that include the following: monocyte chemoattractant protein-1, -2 and -3 (MCP-1, -2, -3), macrophage inflammatory peptides-1α and -1β (MIP-1α and -1β), RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), I309 and several mouse C-C chemokines for which the human homologue has not yet been identified (3). In contrast to C-X-C chemokines, C-C chemokines attract and stimulate a wide spectrum of overlapping and distinct populations of leukocytes (3, 52). This property of C-C chemokines suggests that they play a pivotal role in getting the right immune cell to the infected or inflamed tissue at the right time.

Biological Role and Activities - Similar to C-X-C chemokines, the stimulated monocyte/macrophage is perhaps the richest source of C-C chemokines (1). MCP-1 is stimulated by a variety of stimuli such as phytohemagglutinin, LPS and cytokines like IL-1 and TNFα (1, 53). Activated lymphocytes are another source that secrete RANTES, MIP-1α and -1β although considerably less than monocytes. Indeed, the majority of the chemokines were isolated by subtraction cloning, utilizing stimulated and unstimulated lymphocytes (54). Recently, it has been shown that neutrophils stimulated by various agents secrete MIP-1α or MCP-1 (55). Other cell types that

secrete C-C chemokines include activated endothelial and epithelial cells, fibroblasts, synoviocytes, keratinocytes, smooth muscle and certain tumor cells (1, 54, 56, 57). The pattern of C-C chemokine expression has suggested that chemokines are important in directing specific leukocytes to immune-reactive sites where the appropriate immune responses are initiated and the host response to foreign invasion subsequently amplified by increasing the cell infiltrate at that site (58).

In Vitro Activities - All C-C chemokines induce migration of monocytes but not neutrophils. In addition MCP-1, MCP-2, MCP-3, RANTES and MIP-1α have been shown to induce exocytosis (59). The relative potency of these activities is MCP-1>MCP-3>MCP-2>RANTES>MIP-1α (59). MCP-1 has been reported to elicit the respiratory burst, up-regulation of the adhesion molecule β2-integrin, and an increase in cytokine expression (60-63).

MCP-1 and MCP-3 have been shown to be potent basophil chemoattractants, whereas RANTES and MIP-1α are less potent (64-66). MCP-1 and MCP-3 are stimulators of histamine release while RANTES and MIP-1α are not (64, 66-69). Pretreatment with one chemokine desensitizes the histamine releasing capacity from a second heterologous challenge suggesting that perhaps a single chemokine receptor on basophils is responsible for these activities (70). Finally, RANTES and IL-8 increase adhesiveness of human basophil to cytokine-activated endothelium via β-integrins (71). Recently, RANTES has been shown to attract the mast cell line HMC-1, whereas IL-8, MIP-1α, -1β, and MCP-1 were ineffective (72).

C-C chemokines have been shown to act on eosinophils. *In vitro*, RANTES has been claimed to be the most potent eosinophil chemoattractant (64, 73-75). MCP-3 has been shown to be equivalent to RANTES in terms of attracting eosinophils whereas MIP-1α was shown to be less potent (69, 74). Finally, both RANTES and MCP-3 have been shown to activate eosinophils as assayed by the release of eosinophilic cationic protein (64, 75). The activities of C-C chemokines on basophils and eosinophils have prompted an intense investigation of the role of these pro-inflammatory cytokines in allergic inflammation (*vide infra*).

The effects of C-C chemokines on lymphocytes has been well documented. RANTES is a chemoattractant for memory T cells *in vitro* (76). MIP-1α and -1β chemoattract both naive and activated T cells. MIP-1α has a preference for $CD4^+$ T cells while MIP-1β prefers both $CD8^+$ or $CD4^+$ T cells (77, 78). MCP-1 has been claimed to the most relevant T cell chemoattractant (79). MCP-1, -2 and -3 have been shown to have activities on cloned $CD4^+$ and $CD8^+$ T cell lines, although no response was observed in freshly isolated T cells (80). Finally, certain C-C chemokines have also been shown to increase the adhesiveness of T cells to activated endothelial cells implying that chemokines up-regulate β-integrins (81). Recently, natural killer cells have been shown to migrate in response to RANTES, MIP-1α and MCP-1 (82). Given the spectrum of C-C chemokine activity on different subsets of leukocytes, it has been postulated that the differential expression of the C-C chemokines ensures the appropriate immune response occurs at a localized site, be it a cell-mediated or a humoral response (58).

In Vivo Activities - The *in vitro* activities of C-C chemokines are reflected *in vivo*. For example, MCP-1 injected intradermally, predominantly elicits a monocytic cell infiltrate (60). MCP-2 and MCP-3 have also been shown to have similar activities (83). Injection of RANTES in dogs elicits a monocytic and an eosinophilic infiltrate (84). In one report, the migration of human mononuclear cells in response to RANTES has been shown in SCID mice reconstituted with human peripheral blood leukocytes and provides strong evidence that *in vitro* activities correspond to those *in vivo* (85).

Pathophysiology - MCP-1 has been shown to be constituitively expressed by synovial fibroblasts from RA patients, and its levels are higher in RA joints compared to normal

joints or those from other arthritic diseases (86). MCP-1 is also found in the lungs of patients with idiopathic pulmonary fibrosis and is thought to be responsible for the influx of mononuclear phagocytes and the production of growth factors responsible for stimulating mesenchymal cells and subsequent fibrosis (87). MCP-1 is also involved in the accumulation of monocytes in pleural effusions which is associated with both *Mycobacterium tuberculosis* infection and malignancy (5). The invasion of monocytes into atherosclerotic areas is thought to be due to the presence of MCP-1 as shown by immunohistochemistry of macrophage-rich arterial wall from humans (88, 89). The majority of the MCP-1 probably comes from the smooth muscle cells. Few studies to demonstrate the role of MIP-1α or MIP-1β in human diseases have been published. Most work has been performed in the mouse, e.g. injection into mouse footpad showed neutrophil infiltration and a prostaglandin-independent pyrogenic activity (2). MIP-1α has contradictory myelopoietic-enhancing and stem cell inhibitory activity (SCI), whereas MIP-1β blocks the SCI activity of MIP-1α (2). However, MIP-1α and RANTES are proposed to be involved in allergic inflammatory diseases, such as asthma and atopic dermatitis, by virtue of their eosinophil attractant properties (74, 90). RANTES, for example, has been shown to induce trans-endothelial migration of eosinophils (91). Also, lymph nodes from patients suffering with delayed-type hypersensitivity show an increased production of RANTES which has been linked to the recruitment of macrophages and memory T-cells (92). Finally, a role for chemokines in neurological autoimmune diseases such as multiple sclerosis has been proposed (93).

Receptor/ligand Interactions - Receptor binding analysis indicates the presence of high affinity sites for MIP-1α, -1β, RANTES and MCP-1 on human monocytes (94-98). Studies indicate that at least two monocyte receptors exist, one that predominantly binds MCP-1 and -3 and a second that binds RANTES, MIP-1α and MIP-1β (59). However, there is a complex interaction of chemokines to these receptors since the relative affinities of the individual chemokines to these receptors and the cloned receptor (*vide infra*) are different (59). The proposal that two C-C chemokine receptors are present on monocytes is supported in receptor desensitization studies using the calcium flux assay (59, 70). Similar cross-desensitization studies on basophils and eosinophils indicate the presence of a third receptor, one that binds RANTES and MCP-3 with high affinity (52,64,66). Furthermore, basophils have been postulated to have all three receptors, while eosinophils have the MCP-1 receptor and a second C-C chemokine receptor (52,64,99).

The MCP-1 and the MIP-1α/RANTES (also called the CC-CKR1) receptors have been cloned and like the IL-8 receptors, belong to the GCR superfamily (100-103). Two isoforms of the MCP-1 receptor exist, MCP-1rA and MCP-1rB. Both are the product of the same gene that undergoes differential splicing. The consequence of differential splicing is that the amino acid sequence from the N-terminal to the end of transmembrane domain seven is identical in the two receptors, but the carboxy-termini are different. MCP-1rB binds MCP-1 and -3 with high affinity whereas the properties of the MCP-1rA have not yet been determined (104). The expression of the MCP-1 receptors seems to be restricted to monocytes and is consistent with the biochemically defined MCP-1 receptor on monocytes (59,100).

The cloned MIP-1α/RANTES receptor has all the characteristics of the biochemically defined MIP-1α receptor on monocytes with the exception that it does not bind MIP-1β with high affinity (102,103). Since MIP-1α responses correspond to the cell types expressing this receptor, it is assumed that all biological activities of MIP-1α are mediated through this receptor. However, all the overlapping and distinct C-C chemokine activities cannot be explained by the presence of the MCP-1 receptor or the MIP-1α/RANTES receptors and additional chemokine receptors most likely exist (70). The number and functional responses of C-C chemokine receptors will be more precisely determined when the array of genes encoding the C-C chemokine receptors have been cloned. It will be important to study the cloned receptors in the appropriate

cell type to fully define the functionality of the individual receptors and their role in different disease states and, consequently, in identifying specific receptor antagonists.

THERAPEUTIC INTERVENTIONS.

Pharmacological Modulation - There are few publications on the modulation of the effects of IL-8. The first antagonists were produced by modification of the 72 residue form of IL-8. A series of 26 analogs of IL-8[4-72] were tested. The two most potent analogs were IL-8[6-72] **1** and IL-8[4-72] **2** where Glu-4 and Leu-5 were replaced with Ala-4 and Ala-5. The compounds blocked IL-8 binding and functional activity with IC_{50}'s of 0.3 μM (105). The compounds also inhibited NAP-2 and GROα binding. NMR-derived structure determinations of **1** and **2** failed to provide a clear explanation for the SAR of the series (106).

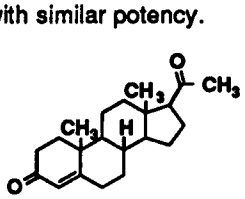
IL-8[6-72]
1

IL-8[4-72]-E4A,L5A
2

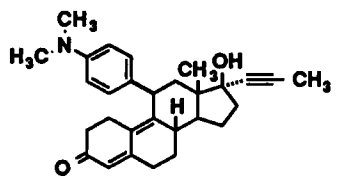
Ac-RRWWCR-NH2
3

Ac-dRdRdWdWdCdRdCNH2
4

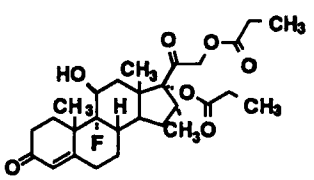
More recently a series of hexa- and hepta-peptides with IL-8 antagonist activity have been prepared. The lead peptide **3** inhibited binding of IL-8 to neutrophils with characteristics consistent with a two-site interaction (IC_{50}'s of 3.6 μM and 58.6 μM) and thus the presence of two receptor populations (107). Truncation of the peptide led to inactive compounds, but the all D-amino acid analog **4** also inhibited IL-8 binding with similar potency.



5

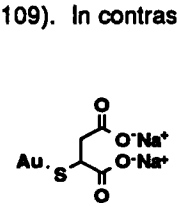


6

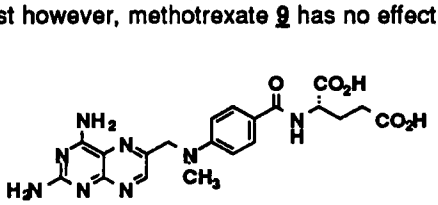


7

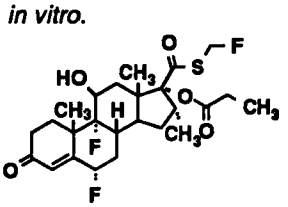
Progesterone **5** has been shown to inhibit the spontaneous production of IL-8 from rabbit uterine cervical fibroblasts as well as IL-1-induced production of IL-8 (108) The effects are blocked by pretreatment with the steroid antagonist mifepristone (RU486, **6**). NSAID's failed to inhibit the production of IL-8 in cultured synoviocytes whereas dexamethasone **7** and gold sodium thiomalate **8** both reduced IL-8 levels (109). In contrast however, methotrexate **9** has no effect *in vitro*.



8



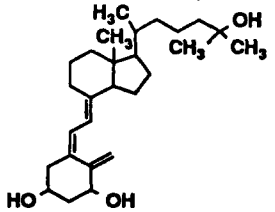
9



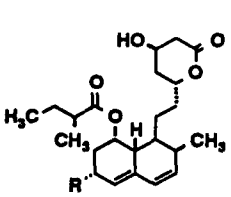
10

Dexamethasone **7** also blocks IL-8 production in cultured airway epithelial cells while fluticasone **10** has been shown to inhibit IL-8 production from cultured nasal epithelial cells (110, 111). 1,25-Dihydroxy vitamin D3 **11** inhibits IL-8 and IL-6 production in human fibroblast cell lines (112). Pretreatment of THP-1 cells with the HMG-CoA reductase inhibitors lovastatin **12** or compactin **13** at 10 μM, under conditions which did not compromise cell viability, reduced (by 50-90%) the ability of the cells to produce IL-8 in response to pro-inflammatory stimuli (113). The addition of

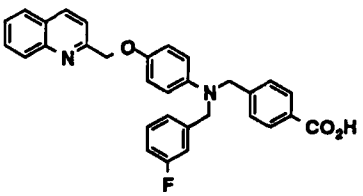
exogenous mevalonate blocked this effect and suggests a dependence of IL-8 production on isoprenoid production. ETH615 **15**, a known 5-LO inhibitor, inhibits LPS-induced IL-8 production and IL-8 directed chemotaxis of human neutrophils (114).



11



12 R=CH_3; **13** R =H



15

Very few examples of C-C chemokine inhibitors have been described. Similar to IL-8 analogs, truncation of the N-termini of MCP-1 results in antagonistic analogs (115, 116). These analogs were shown to interact with the normal MCP-1 monomers and render the heterodimer inactive, i.e. the analogs were acting as dominant-negative suppressers (115, 116). These analogs have the potential to utilize gene therapy as a mode of modulating MCP-1 activity *in vivo* especially in diseases like atherosclerosis where targeted expression of such analogs is desired.

A different approach to target chemokines is suggested by the proposal that chemokine activity *in vivo* occurs by interaction of chemokines with proteoglycans on the surface of the endothelium (117,118). In this model, a solid phase gradient is proposed to exist on an inflamed endothelium where chemokines are held in place by proteoglycan. Several studies have supported this model where the activity of chemokines *in vitro* is modulated in the presence of proteoglycans and affinities between chemokines and proteoglycans have been defined (81, 119, 120). Disrupting the proteoglycan/chemokine interaction has the potential of modulating chemokine function *in vivo* (119).

Another approach to the modulation of chemokine activity *in vivo* is based on agonist actions that will down-regulate chemokine receptor and thus give rise to leukocyte unresponsiveness. For example, transgenic mice overexpressing IL-8 have been shown to have a phenotype that manifests in neutrophil unresponsiveness (121). This has been also shown clinically where inhalation of IL-8 reverses hyperreactivity and blocks allergen-induced late phase reactions (122).

Finally, the biological activity of chemokines has been exploited for cancer therapy where chemokine overexpression in cancer cells has had dramatic effects on tumor growth *in vivo*. Several studies have shown that transfection of chemokine genes into tumor cells results in tumor growth regression in animal models implanted with such chemokine expressing tumor cells e.g. expression of MCP-1 and TCA-3, the mouse homologue of I309, in different tumor cells results in impaired tumor growth in syngeneic animals (123-126). Thus, these results support the use of chemokines as vaccines in cancer therapy.

Conclusion - It is apparent that the chemokines play a central role in leukocyte trafficking. Therefore, they are likely to be very important mediators in disease processes that depended on leukocyte infiltration and damage. However, there is a tremendous redundancy in the system: each subset of leukocyte is affected by at least three if not more chemokines. Such overlapping yet at times distinct biological properties of chemokines could be important for the fine tuning and regulation of the immune response to an invading infectious agent. Initial observations with neutralizing antibodies and gene knock out animals provide overwhelming support that inactivating chemokine responses can be efficacious in certain inflammatory disease models. As receptor/ligand interactions are fully defined and signal transduction

pathways elucidated, additional defined molecular targets will be identified. These targets will provide a rich selection of ways to modulate chemokine activity by pharmacological agents.

REFERENCES

1. M. Baggiolini, B. Dewald and B. Moser, Adv.Immunol., 55, 97 (1994).
2. DJ. Kelvin, DF. Michiel, J.A. Johnston, A.R. Lloyd, H. Sprenger, J.J. Oppenheim and J.M. Wang, J.Leukoc.Biol., 54, 604 (1993).
3. M.D. Miller and M.S. Krangel, Crit.Rev.Immun., 12, 17 (1992).
4. J. Van Damme in "The Cytokine Handbook," Vol.A.Thomson, Eds., Academic Press, 1994, p.185.
5. R.M. Strieter, A.E. Koch, V.B. Antony, R.J. Fick, T.J. Standiford and S.L. Kunkel, J.Lab.Clin.Med., 123, 183 (1994).
6. T.A.Springer, Cell, 76,301 (1994)
7. R.W. McCain, E.P. Holden, T.R. Blackwell and J.W. Christman, Am.J.Respir.Cell.Mol.Biol., 10, 651 (1994).
8. A. Walz,R. Burgener,B. Car,M. Baggiolini, S.L. Kunkel and R.M. Strieter, J.Exp.Med., 174, 1355 (1991).
9. G. Cacalano, J. Lee, K. Kikly, A.M. Ryan, M.S. Pitts, B. Hultgren, W.I. Wood and M.W. Moore, Science, 265, 682 (1994).
10. L. Kemeny, A.S. Kenderessy, E. Olasz, G. Michel, T. Ruzicka, B. Farkas and A. Dobozy, Eur.J.Pharmacol., 258, 269 (1994).
11. M. Seitz, B. Dewald, N. Gerber and M. Baggiolini, J.Clin.Invest., 87, 463 (1991).
12. M. Rampart, A.G. Herman, B. Grillet, G. Opdenakker and J.Van Damme, Lab.Invest., 66, 512 (1992).
13. J.P. Lynch, T.J. Standiford III, M.W. Rolfe, S.L. Kunkel and R.M. Strieter, Am.Rev.Respir.Dis., 145, 1433 (1992).
14. S.C. Donnelly, R.M. Strieter, S.L. Kunkel, A. Walz, C.R. Robertson, D.C. Carter, I.S. Grant, A.J. Pollok and C. Haslett, Lancet, 341, 643 (1993).
15. V.C. Broaddus, C.A. Hebert, R.V. Vitangcol, J.M. Hoeffel, M.S. Bernstein and A.M. Boylan, Am.Rev.Respir.Dis., 146, 825 (1992).
16. C. Marty, B. Misset, F. Tamion, C. Fitting, J. Carlet and J.M. Cavaillon, Crit.Care Med., 22, 673 (1994).
17. K.S. Maxwell, J.E. Fitzgerald, J.A. Burleson, G. Leonard, R. Carpenter and D.L. Kreutzer, Laryngoscope 104, 989 (1994).
18. A.S. Mistchenko, R.A. Diez, A.L. Mariani, J. Robaldo, A.F. Maffey, B.G. Bayley and S. Grinstein, J.Pediatr. 124, 714 (1994).
19. K. Mitsuyama, A. Toyonaga, E. Sasaki, K. Watanabe, H. Tateishi, T. Nishiyama, T. Saiki, H. Ikeda, O. Tsuruta and K. Tanikawa, Clin.Exp.Immunol., 96, 432 (1994).
20. N. Sekido, N. Mukaida, A. Harada, I. Nakanishi, Y. Watanabe and K. Matsushima, Nature, 365, 654 (1993).
21. M. Hogan, B. Sherry, C. Ritchlin, M. Fabre, R. Winchester, A. Cerami and R. Bucala, Cytokine, 6, 61 (1994).
22. W. Tettelbach, L. Nanney, D. Ellis, L. King and A. Richmond, J.Cutan.Pathol., 20, 259 (1993).
23. E.N. Unemori, E.P. Amento, E.A. Bauer and R. Horuk, J.Biol.Chem., 268, 1338 (1993).
24. Q.C. Cheng, J.H. Han, H.G. Thomas, E. Balentien and A. Richmond, J.Immunol., 148, 451 (1992).
25. R. Horuk, D.G. Yansura, D. Reilly, S. Spencer, J. Bourell, W. Henzel, G. Rice and E. Unemori, J.Biol.Chem., 268, 541 (1993).
26. A. Walz in "Cytokines," Vol.4, M.Baggiolini and C.Sorg, Eds., Karger, Basel, 1992, p.77.
27. B. Moser, C. Schumacher, V. von Tscharner, I. Clark-Lewis and M. Bagiolini, J.Biol.Chem., 266, 10666 (1991).
28. C. Schumacher, I. Clark-Lewis, M. Baggiolini and B. Moser, Proc.Natl.Acad.USA, 89, 10542 (1992).
29. W. Schnitzel, B. Garbeis, U. Monschein and J. Besemer, Biochem.Biophys.Res.Commun., 180, 301 (1991).
30. W.E. Holmes, J. Lee, W.-J. Kuang, G.C. Rice and W.I. Wood, Science, 253, 1278 (1991).
31. P.M. Murphy and H.L. Tiffany, Science, 253, 1280 (1991).
32. J. Lee, R. Horuk, G.C. Rice, G.L. Bennett, T. Camerato and W.I. Wood, J.Biol.Chem, 267, 16283 (1992).
33. G.J. LaRose, K.M. Thomas, M.E. Kaufmann, R. Mark, M. White, L. Taylor, G. Gray, D. Witt and J. Navarro, J.Biol.Chem, 267, 25402 (1992).
34. R. Gayle, P.R. Sleath, S. Srinivason, C.W. Birks, K.S. Weerawarna, D.P. Cerretti, C.J. Kozlosky, N. Nelson, B.T. Vanden and M.P Beckmann, J.Biol.Chem, 268, 7283 (1993).
35. G.M. Clore, E. Appella, M. Yamada, K. Matsushima and A.M. Gronenborn, J.Biol.Chem., 264, 18907 (1989).

36. E.T. Baldwin, I.T. Weber, R. St.Charles, X.J.-Xuan., E. Appella, M. Yamada, K. Matsushima, B.F.P. Edwards, G.M. Clore, A.M. Gronenborn and A. Wlodawer, Proc.Natl.Acad.Sci.U.S.A., 88 (1991).
37. G.M. Clore, E. Appella, M. Yamada, K. Matsushima and A.M. Gronenborn, Biochem., 29, 1689 (1990).
38. C.A. Hebert, R.Vitangcol and J.B. Baker, J.Biol.Chem, 266, 18989 (1991).
39. I. Clark-Lewis, B. Dewald, M. Loetscher, B. Moser and M. Baggiolini, J Biol Chem, 269, 16075 (1994).
40. I. Clark-Lewis, C. Schumacher, M. Baggiolini and B. Moser, J.Biol.Chem., 266, 23128 (1991).
41. B. Dewald, B. Moser, B. Barella, C. Schumacher, M. Baggiolini and I. Clark-Lewis, Immunol.Lett.., 32, 81 (1994).
42. I. Clark-Lewis, B. Dewald, T. Geiser, B. Moser and M. Baggiolini, Proc.Natl.Acad.Sci.U S A, 90, 3574 (1993).
43. I.U. Schraufstatter, D.S. Barritt, M. Ma, Z.G. Oades and C.G. Cochrane, J.Immunol., 151, 6418 (1993).
44. C.A. Hebert, A. Chuntharapai, M. Smith, T. Colby, J. Kim and R. Horuk, J.Biol.Chem, 268, 18549 (1993).
45. S.R. Leong, R.C. Kabakoff and C.A. Hebert, J.Biol.Chem., 269, 19343 (1994).
46. R.W. Kupper, B. Dewald, K.H. Jakobs, M. Baggiolini and B. Moser, Biochem.J., 282, 429 (1992).
47. K.B. Bacon, D.G. Quinn, J.P. Aubry and R.D. Camp, Blood, 81, 430 (1993).
48. R.J. Smith, L.M. Sam, K.L. Leach and J.M. Justen, J.Leukocyte Biol., 52, 17 (1992).
49. J.A. Johnston, D.K. Ferris, J.M. Wang, D.L. Longo, J.J. Oppenheim and D.J. Kelvin, European J. Immunol., 24, 2556 (1994).
50. T. Pozzan, D.P. Lew, C.B. Wollheim and R.Y. Tsien, Science, 221, 1413 (1983).
51. D. Wu, G.J. LaRosa and M.I. Simon, Science, 261, 101 (1993).
52. M. Baggiolini and C.A. Dahinden, Immunol.Today, 15, 127 (1994).
53. F. Colotta, A. Borre, J.M. Wang, M. Tattanelli, F. Maddalena, N. Polentarutti, G. Peri and A. Mantovani, J.Immunol., 148, 760 (1991).
54. T.J. Schall, Cytokine, 3, 165 (1991).
55. T.C. Burn, M.S. Petrovick, S. Hohaus, B.J. Rollins and D.G. Tenen, Blood, 84, 2776 (1994).
56. Z. Brown, M.E. Gerritsen, W.W. Carley, R.O.M. Strieter, S.L. Kunkel and J. Westwick, Am.J.Path., 145, 913 (1994).
57. A. Mantovani, Lab.Invest., 71, 5 (1994).
58. T.J. Schall and K.B. Bacon, Curr.Opin.Immunol., 6, 865 (1994).
59. M. Uguccioni, M. D'Apuzzo, M. Loetscher, B. Dewald and M. Baggiolini, Eur.J.Immunol., 25, 64 (1995).
60. C.O.C. Zachariae, A.O. Anderson, H.L. Thompson, E. Appella, A. Mantovani, J.J. Oppenheim and K. Matsushima, J.Exp.Med., 171, 2177 (1990).
61. B.J. Rollins, A. Walz and M. Baggiolini, Blood, 78, 1112 (1991).
62. Y. Jiang, D.I. Beller, G. Frendl and D.T. Graves, J.Immunol., 148, 2423 (1992).
63. Y. Jiang, J.F. Zhu, F.W. Luscinskas and D.T. Graves, Am.J.Physiol.Cell Physiol, 267, 37 (1994).
64. C.A. Dahinden, T. Geiser, T. Brunner, T.V.von, D. Caput, P. Ferrara, A. Minty and M. Baggiolini, J.Exp.Med., 179, 751 (1994).
65. S.C. Bischoff, M. Krieger, T. Brunner and C.A. Dahinden, J.Exp.Med., 175, 1271 (1992).
66. S.C. Bischoff, M. Krieger, T. Brunner, A. Rot, T.V. von, M. Baggiolini and C.A. Dahinden, Eur.J.Immunol., 23, 761 (1993).
67. R. Alam, P. Forsythe, S. Stafford, J. Heinrich, R. Bravo, P. Proost and D.J. Van, J.Immunol, 153, 3155 (1994).
68. R. Alam, P. Forsythe, S. Stafford, P. Proost and D.J. Van, Expt. Biol., 8 (1994).
69. P. Kuna, S.R. Reddigari, T.J. Schall, D. Rucinski, M. Sadick and A.P. Kaplan, J.Immunol., 150, 1932 (1993).
70. T.J. Schall, J.Y. Mak, D. DiGregorio and K. Neote, Adv.Exp.Med.Biol., 351, 29 (1993).
71. K.B. Bacon, R.L. Flores, J.P. Aubry, T.N.C. Wells and C.A. Power, Immunol., 82, 473 (1994).
72. G. Nilsson, J.H. Butterfield, K. Nilsson and A. Siegbahn, J.Immunol., 153, 3717 (1994).
73. Y. Kameyoshi, A. Dorschner, A.I. Mallet, E. Christophers and J.M. Schroeder, J.Exp.Med., 176, 587 (1992).
74. A. Rot, M. Krieger, T. Brunner, S.G. Biscoff, T.J. Schall and C.A. Dahinden, J.Exp.Med., 176, 1489 (1992).
75. R. Alam, S. Stafford, P. Forsythe, R. Harrison, B.M.A. Lett and J.A. Grant, 49th Ann.Am.Acad.Allergy.Immunol., Chicago, Illinois, Usa, March (1993).
76. T.J. Schall, K. Bacon, K.J. Toy and D.V. Goeddel, Nature, 347, 669 (1990).
77. T.J. Schall, K. Bacon, R.D.R. Camp, J.W. Kaspari and D.V. Goeddel, J.Exp.Med., 177, 1821 (1993).
78. D.D. Taub, K. Conlon, A.R. Lloyd, J.J. Oppenheim and D.J. Kelvin, Science., 60, 355 (1993).
79. M.W. Carr, S.J. Roth, S.S. Rose, E. Luther and T.A. Springer, Keystone Symposium on Lymphocyte Activation, Keystone, Colorado, USA, April (1994).
80. P. Loetscher, M. Seitz, L.I. Clark, M. Baggiolini and B. Moser, FASEB.J, 8, 1055 (1994).

81. T. Tanaka, D.H. Adams, S. Hubscher, H.Hirano, U.Siebenlist and S.Shaw, Nature, 361, 79 (1993).
82. A.A. Maghazachi, A. Al-Aoukaty and T.J. Schall, J.Immunol., 153, 4969 (1994).
83. J. Van Damme, P. Proost, J.-P. Lenaerts and G. Opdenakkerm, J.Med.Chem., 176, 59 (1992).
84. R. Meurer, R.G. Van, W. Feeney, P. Cunningham, D.J. Hora, M.S. Springer, D.E. MacIntyre and H. Rosen, J.Exp.Med., 178, 1913 (1993).
85. W.J. Murphy, D.D. Taub, M. Anver, K. Conlon, J.J. Oppenheim, D.J. Kelvin and D.L. Longo, Eur.J.Immunol., 24, 1823 (1994).
86. A.E. Koch, S.L. Kunkel, L.A. Harlow, B. Johnson, H.L. Evanoff, G.K. Haines, M.D. Burdick, R.M. Pope and R.M. Strieter, J.Clin.Invest, 90, 772 (1992).
87. H.N. Antoniades, J. Neville-Golden, T. Galanopoulos, R.L. Kradin, A.J. Valente and D.T. Graves, Proc.Natl.Acad.Sci.USA, 89, 5371 (1992).
88. S. Yla-Herttuala, B.A. Lipton, M.E. Rosenfeld, T. Srkioja, T. Yoshimura, E.J. Leonard, J.L. Witztum and D. Steinberg, Proc.Natl.Acad.Sci.USA, 88, 5252 (1991).
89. N.A. Nelken, S.R. Coughlin, D. Gordon and J.N. Wilcox, J.Clin.Invest., 88, 1121 (1991).
90. A. Kapp, K.G. Keck, W. Czech and E. Schopf, J.Invest.Dermatol., 102, 906 (1994).
91. M. Ebisawa, T. Yamada, C. Bickel, D. Klunk and R.P. Schleimer, J.Immunol., 153, 2153 (1994).
92. O. Devergne, K.A. Marfaing, T.T. Schall, R.M. Leger, M. Sadick, M. Peuchmaur, M.C. Crevon, T. Kim, P. Galanaud and D. Emilie, J.Exp.Med., 179, 1689 (1994).
93. M. Tani and R.M. Ransohoff, Brain Pathol., 4, 135 (1994).
94. J.M. Wang, B. Sherry, M.J. Fivash, D.J. Kelvin and J.J. Oppenheim, J.Immunol., 150, 3022 (1993).
95. J.M. Wang, D.W. Mcvicar, J.J. Oppenheim and D.J. Kelvin, J.Exp.Med., 177, 699 (1993).
96. G. Van Riper, S. Siciliano, P.A. Fischer, R. Meurer, M.S. Springer and H. Rosen, J.Exp.Med., 177, 851 (1993).
97. T. Yoshimura and E.J. Leonard, J.Immunol., 145, 292 (1990).
98. Y. Yamamaura, T. Hattori, Y. Ohmoto and K. Takatsuki, Int.J.Hematol., 55, 131 (1992).
99. C.A. Dahinden, M. Krieger, T. Brunner and S.C. Bischoff, Adv.Exp.Med.Biol., 351, 99 (1993).
100. I.F. Charo, S.J. Myers, A. Herman, C. Franci, A.J. Connolly and S.R. Coughlin, Proc.Natl.Acad.Sci.USA, 91, 2752 (1994).
101. H. Nomura, B.W. Nielsen and K. Matsushima, Int.Immunol., 5, 1239 (1994).
102. J.L. Gao, D.B. Kuhns, H.L. Tiffany, D. Mcdermott and P.M. Murphy, J.Exp.Med., 177, 1421 (1993)
103. K. Neote, D. DiGregorio, J.Y. Mak, R. Horuk and T.J. Schall, Cell, 72, 415 (1993).
104. S.J. Myers, L.M. Wong and I.F. Charo, J.Biol.Chem..270, 5786 (1995)
105. B. Moser, B. Dewald, L. Barella, C. Schumacher, M. Baggiolini and L.I. Clark, J.Biol.Chem., 268, 7125 (1993).
106. K. Rajarathnam, L.I. Clark and B.D. Sykes, Biochem., 33, 6623 (1994).
107. S. Hayashi, A. Kurdowska, E.J. Miller, M.E. Albright, B.E. Girten and A.B. Cohen, J.Immunol., 154, 814 (1995).
108. A. Ito, K. Imada, T. Sato, T. Kubo, K. Matsushima and Y. Mori, Biochem.J. (1994).
109. P. Loetscher, B. Dewald, M. Baggiolini and M. Seitz, Cytokine, 6, 162 (1994).
110. O.J. Kwon, B.T. Au, P.D. Collins, J.N. Baraniuk, I.M. Adcock, K.F. Chung and P.J. Barnes, Immunol., 81, 389 (1994).
111. M. Ohnishi, R. Takizawa, K. Ohkubo, K. Yokosima, M. Okuda and T. Yagi, Arerugi, 43, 441 (1994).
112. M.D. Srviastava, H. DeLuca and J.L. Ambrus, Res Commun Chem Pathol Pharmacol, 83, 145 (1994).
113. R. Terkeltaub, J. Solan, M.J. Barry, D. Santoro and G.M. Bokoch, J.Leukoc.Biol., 55, 749 (1994).
114. M. Kristensen, T. Jinquan, M.K. Thomsen, C. Zachariae, K. Paludan, R.I. Ahnfelt, K. Matsushima, P.K. Thestrup and C.G. Larsen, Exp.Dermatol., 2, 165 (1993).
115. J.Z. Yu, B.J. Rutledge and B.J. Rollins, J.Biol.Chem., 269, 15918 (1994).
116. J.-H. Gong and I. Clark-Lewis, J.Exp.Med., 181, 631 (1995).
117. A. Rot, Immunol.Today, 13, 291 (1992).
118. Y. Tanaka, D.H. Adams and S. Shaw, Immunol.Today, 14, 111 (1993).
119. D.P. Witt and A.D. Lander, Curr.Biol., 4, 394 (1994).
120. C.J. Wiedermann, E. Kowald, N. Reinisch, C.M. Kaehler, L.I. Von, J.M. Pattison, P. Huie, R.K. Sibley, P.J. Nelson and A.M. Krensky, Curr.Biol., 3, 735 (1993).
121. W.S. Simonet, T.M. Hughes, H.Q. Nguyen, L.D. Trebasky, D.M. Danilenko and E.S. Medlock, J.Clin.Invest., 94, 1310 (1994).
122. R.H. Fisher, L.B. Atkinson and W.J. Metzger, Ann.Allergy, 70, 63 (1993).
123. A.D. Luster and P. Leder, J.Exp.Med., 178, 1057 (1993).
124. S. Huang, R.K. Singh, K. Xie, M. Gutman, K.K. Berry, C.D. Bucana, I.J. Fidler and E.M. Bar, Cancer Immunol.Immunother., 39, 231 (1994).
125. J. Laning, H. Kawasaki, E. Tanaka, Y. Luo and M.E. Dorf, J.Immunol., 153, 4625 (1994).
126. B. Bottazzi, S. Walter, D. Govoni, F. Colotta and A. Mantovani, J.Immunol., 148, 1280 (1992).

SECTION V. TOPICS IN BIOLOGY

Editor: John C. Lee
SmithKline Beecham Pharmaceuticals
King of Prussia, Pennsylvania 19406

Chapter 23. Gene Therapy: Progress, New Directions, and Issues

Leonard E. Post
Parke-Davis Pharmaceutical Research, Ann Arbor, Michigan 48105

Introduction - Gene therapy is a topic that encompasses an extremely wide range of techniques and applications. It includes any technique to introduce exogenous DNA into cells for a therapeutic purpose. There are so many different types of gene therapy delivery vehicles, and so many different applications emerging, that the field could perhaps justify a new series entitled "Annual Reports in Gene Therapy". This chapter will make no attempt to provide a comprehensive review of the field, but instead give a few examples that include publication of clinical results; new areas were gene therapy provides an innovative drug delivery system; and a few issues facing the field.

For background in gene therapy, the reader is referred to a recent volume of reviews (1), and various reviews of vector systems (2,3,4). For a complete summary of gene therapy clinical trials underway and updates on their progress, the databases of the NIH Office of Recombinant DNA Activities (ORDA) are regularly published in the journal *Human Gene Therapy*.

Clinical Results - The first human gene therapy was in 1990, and the publication of clinical results has begun to appear. Although in most cases the trials are not far enough along to claim clinical benefits to the patients, it has been established that gene transfer to humans can be accomplished, the genes can be expressed, and the gene products can have effects.

Nabel, *et al.*, (5) reported the early results of a clinical trial in which DNA encoding major histocompatibility complex class I protein B7 was injected directly into melanoma nodules. The goal of this treatment was to enhance the immune response to tumor. This trial was ground breaking in several respects. It was the first trial to introduce the transferred gene directly into a patient, as opposed to removing the cells and transfection *ex vivo*. It was the first example of a non-viral vector system; i.e., instead of a recombinant virus, the gene was delivered as a purified DNA in a cationic liposome. Of the first five patients treated, there were no adverse events associated with the gene transfer. In all five patients, evidence demonstrated HLA-B7 gene expression in the tumor. Cell-mediated

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

immune response to B7 was obtained, as well as increase in cytotoxic T cells responsive to tumor. Particularly encouraging, one of the patients demonstrated a regression of not only the injected nodule, but also distant nodules. In one patient, a pulmonary nodule was injected via a vascular catheter (6). A second generation of this treatment involves an improved vector for more efficient expression of B7, and a new cationic lipid that allows higher concentrations of DNA to be delivered, is now underway (7). Other trials are testing this therapy for melanoma (8), renal cancer (9), and hepatic metastases of colon cancer have also been initiated (10).

Grossman, *et al.* (11) reported the first results from a trial in which a gene encoding low density lipoprotein (LDL) receptor was introduced into hepatocytes of a patient with familial hypercholesterolemia (FH). FH is a condition where this receptor is defective, leading to extremely high levels of LDL cholesterol and severe atherosclerosis. The treatment involves partial hepatectomy, *in vitro* culture of hepatocytes, transfection of the hepatocytes with a retroviral vector encoding LDL receptor, then re-introduction of the transfected hepatocytes via the portal vein. This procedure was accomplished without adverse events. In the single patient reported, a substantial reduction in serum LDL was observed for months after the procedure. Although the interpretation of this result has been debated (12), the safety and apparent efficacy observed on the first patient is encouraging.

Cystic fibrosis (CF) has been the genetic disease receiving the most intensive attention by gene therapy approaches, and results from three clinical trials have been published. The cystic fibrosis transmembrane regulator (CFTR) gene has been engineered into adenovirus vectors. Since adenovirus normally infects the respiratory tract, the natural biology of the virus may be well-suited for delivery of CFTR to the respiratory tract. The initial study in the nose demonstrated that the CFTR gene could be transferred to man via adenovirus and that this treatment could correct the chloride transport defect of cystic fibrosis in the treated region of the nasal epithelium (13). Introduction of the adenovirus encoding CFTR into patients' lungs has also been demonstrated to successfully transfer the CFTR gene, and to be safe at low doses (14). However, this trial also reported pulmonary complications in a single patient treated with a higher dose, that may be due to release of IL6 induced by the adenovirus (15). In another approach, the CFTR gene was introduced into the nose via cationic liposomes, also demonstrating gene transfer and transient correction of the chloride transport defect (16). Significant challenges remain to achieve gene transfer for sustained periods of time, and in sufficient areas of the respiratory epithelium to achieve clinical benefit. Additional trials are underway with adenovirus vectors involving multiple administration and alternative delivery methods. A trial with an alternative cationic liposome is also underway (17).

New Directions for Gene Therapy - Initially gene therapy was envisioned as a way to correct genetic defects. It is becoming increasingly clear that the technology to introduce genes into patients will have much broader implications. In fact, gene therapy is a versatile delivery vehicle for therapeutic proteins or RNAs. This section will summarize a few of the many new directions emerging for gene therapy.

There are a variety of strategies for gene therapy of cancer. One intriguing strategy is the introduction into tumor cells of a gene that can locally activate a prodrug (18). The strategy was first applied clinically by using the herpes simplex virus thymidine kinase (tk) gene to activate ganciclovir. Ganciclovir is not phosphorylated by mammalian thymidine kinases, but is efficiently phosphorylated to a form that can inhibit DNA synthesis via herpesviral thymidine kinases. Cells producing the tk-encoding retroviral vector were injected intra-tumorally into rat gliomas, and after several days of *in vivo* infection the gliomas responded to systemic administration of ganciclovir (19, 20). Apparently tumor cells in addition to those transfected with the vector die in response to ganciclovir, the so-called "bystander effect". This treatment has entered clinical testing in glioblastoma patients (21), and the initial results gave sufficient confidence in safety to justify expansion to multiple treatments via an Omaya reservoir (22), into pediatric patients (23), and a treatment for leptomeningeal carcinomatosis (24). In other preclinical studies with this system, there are suggestions that killing of tumors via the tk/ganciclovir protocol can result in systemic antitumor immunity (25). More recent results delivering the tk gene into a tumor via an adenoviral vector, followed by ganciclovir treatment showed impressive efficacy in the rat glioma model (26, 27). Adenoviral vectors provide an alternative to the retroviral producer cells for delivery of the thymidine kinase gene, and this approach is rapidly progressing toward clinical testing. Other examples of strategies to deliver genes to activate prodrugs into anti-tumor cytotoxic agents include the cytosine deaminase gene that activates 5-fluorocytosine to 5-fluorouracil (28,29); and the *P450 2B1* gene to activate cyclophosphamide (30).

The application of gene therapy to cardiovascular disease has been recently reviewed by E. G. Nabel (31), who pioneered the delivery of DNA via a vascular catheter (32). 1994 saw the initiation of the first clinical trial of catheter delivery of DNA (33). In this protocol, a purified DNA encoding vascular endothelial growth factor is introduced to an artery wall via a hydro-gel coated angioplasty balloon (34), the goal being to stimulate angiogenesis in severe peripheral vascular disease. In the past year, two studies described efficacy of catheter-delivered genes in preventing restenosis after balloon angioplasty in the pig model. The first of these used catheter delivery of an adenovirus carrying the herpes simplex virus thymidine kinase gene to the vascular wall at the site of injury, followed by systemic treatment with ganciclovir (35). Via a mechanism exactly as explained above for anti-tumor treatment, the thymidine kinase encoded by the transferred gene activated the ganciclovir in the transfected cells to an active form that is cytotoxic to proliferating cells. The result was a reduction in the proliferative lesion following injury. In a cytostatic strategy, an adenovirus containing a non-phosphorylated (i.e., constituitively active) mutant of the retinoblastoma gene was introduced into the vessel wall via catheter after balloon angioplasty (36). The result was reduction of the proliferative response to injury. In another approach to restenosis, in the rat carotid model the gene for endothelial cell nitric oxide synthetase was found to be efficacious (37).

Gene therapy for AIDS has also reached the clinic in several forms, and a variety of additional strategies are being developed preclinically (reviewed in 38). One example is the use of a dominant negative mutant of HIV rev. A dominant negative mutant is an altered version of a gene that produces a product that is itself non-functional, and in addition interferes with function of the natural protein. HIV rev is a protein needed for proper expression of HIV's spliced mRNAs, and is essential for virus replication. When a mutant of rev, called revM10, is introduced into human T cells they are protected from HIV infection (39). A clinical protocol has begun where CD4-positive T cells from an HIV-infected patient are bombarded with gold particles coated with purified revM10 DNA, and the cells are then subsequently re-introduced to the subject (40). The goal of this study is to evaluate safety of revM10 and determine whether this gene can prolong the survival of transfected T cells in the HIV-infected person.

Other than the brain tumor protocols discussed above, the use of gene therapy in the brain has not yet reached the point of a clinical trial. However, there are a wide variety of approaches and targets for application of gene therapy in neurology (reviewed in 41, 42). A particularly exciting prospect is the introduction of genes encoding dopamine synthetic enzymes into the brains of Parkinson's disease patients. The goal would be to obtain the effect of transplantation of dopamine-producing cells without the logistical problems associated with sourcing of appropriate cells. A recent report described the insertion of the tyrosine hydroxylase gene into an adeno-associated virus vector, and stereotactic injection of the vector into the striatum of rats with symptoms of Parkinson's disease (43). This report described not only sustained expression of the transfected gene, but also resulted in some phenotypic improvement as measured by apomorphine-induced turning.

Another application of gene therapy would be to introduce genes encoding anti-inflammatory proteins into joints as a treatment for arthritis. Two different approaches have been used to introduce the gene for IL1 receptor antagonist protein (IRAP) into the knee joints of rabbits. In one approach, synoviocytes were transfected *ex vivo* with a retroviral vector encoding IRAP, and then re-introduced into a joint (44, 45). In the other approach, an adenovirus carrying the IRAP gene was administered via intraarticular injection (46). Both approaches resulted in IRAP production, and protection from IL1 challenge to the joint.

Issues - DNA can clearly be added and expressed in man through a variety of means, and the potential applications are many. The pace at which these applications become part of the future of medicine depends on resolution of various issues that surround the safety of gene therapy. To date, after treatment of hundreds of patients, there have been few reports of adverse events and most of these are unrelated to the gene transfer *per se*.

A general question about gene therapy is the question of the fate of added DNA. In some cases, such as adenoviral or cationic liposome delivery, the DNA appears to disappear after days or weeks. However, even in these cases, the extent of integration into the cellular

chromosomes is probably not zero and has not been carefully quantitated. For some vectors systems, such as retrovirus or adeno-associated virus, integration into the chromosome is a key feature and provides the possibility of long term gene expression. Integration of DNA offers the theoretical possibility of disruption of tumor suppressor genes, although the quantitative degree of this risk is probably small. The observation that adeno-associated virus integrates into a specific chromosomal site (47) offers promise that such targeting is possible, although to date this specificity has not been achieved with any vector system. One approach to the concern that transfected cells might become tumorigenic is incorporation of a "suicide gene" such as herpes simplex thymidine kinase, which would render the transfected cells susceptible to drugs like ganciclovir (48, 49).

Individual vector systems have their own safety issues. Production lots of retroviral vectors must be stringently monitored for replication competent retrovirus (50), which could itself be pathogenic or tumorigenic. The observed proinflammatory nature of adenoviruses must be carefully addressed in clinical settings where animal models may not be predictive (e.g., 15). At high doses, even debilitated adenovirus vectors may be cytotoxic (51).

One disadvantage of current gene therapy systems is that the expression of the introduced genes is uncontrolled. In every clinical protocol to date, the delivered gene is expressed from a strong constitutive promoter, which expresses the gene until the added DNA is lost or somehow inactivated. There are a variety of strategies in development that involve control of gene expression by small organic molecules. The bacterial tetracycline operon repressor has been engineered to allow control of gene expression by tetracycline, and this system that has been demonstrated to work *in vivo* (52). An engineered progesterone receptor activates genes in response to very low levels of progesterone antagonists such as RU486, and this system has also recently been show to work in transplanted cells in rats (53). The approach of using small molecules to dimerize signaling proteins (54) is also being developed as a way to control genes introduced by vectors. These and related directions of research offer an exciting possibility that gene therapy will involve addition of the therapeutic gene, plus a system to control it. Then dosing with small molecules may control the pharmacology that is delivered via the genes.

Summary - There is now little doubt that gene therapy in humans is feasible. This review mentions just a few of the clinical trials of gene therapy where published results are available. Over a hundred protocols have been approved by the Recombinant DNA Advisory Committee which oversees NIH-supported gene therapy in the United States, and additional gene therapy trials are ongoing in many countries around the world. The list of clinical targets for potential gene therapy approaches includes examples from every branch of medicine, and a short review such as this certainly does not do justice to the many creative approaches that are being pursued. The reader is encouraged to track the literature in the coming years as the issues surrounding gene therapy are addressed and the applications continue to broaden.

Postscript. With the view that most readers of this volume will be medicinal chemists, it is worth explicitly noting the impending impact of medicinal chemistry on the field of gene therapy. The development of non-viral delivery systems beyond the present early stages will require creative medicinal chemistry. Strategies such as genes to activate prodrugs, and use of small molecules to control expression of exogenous genes, offer additional areas for collaboration between medicinal chemists, molecular biologists, and pharmacologists.

References

1. Gene Therapeutics, J. Wolff, Editor, Birkhäuser, Boston, M.A., 1994.
2. R. C. Mulligan, Science, 260, 926 (1993).
3. D. Jolly, Cancer Gene Ther, 1, 51 (1994).
4. H. Farhood, X. Gao, K. Son, Y-Y Yang, J. S. Lazo, L. Huang, J. Barsoum, R. Bottega, and R. M. Epand, Ann N Y Acad Sci, 716, 23 (1994).
5. G. J. Nabel, E. G. Nabel, Z-Y Yang, B. A. Fox, G. E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon, and A. E. Chang, Proc Natl Acad Sci U S A, 90, 11307 (1993).
6. E. G. Nabel, Z. Yang, D. Muller, A. E. Chang, X. Gao, L. Huang, K. J. Cho, and G. J. Nabel, Hum Gene Ther, 5, 1089 (1994).
7. G. J. Nabel, A. E. Chang, E. G. Nabel, G. E. Plautz, W. Ensminger, B. A. Fox, P. Felgner, S. Shu, and K. Cho, Hum Gene Ther, 5, 57 (1994).
8. E. Hersh, E. Akporiaye, D. Harris, A. T. Stopeck, E. C. Unger, J. A. Warnecke and S. A. Kradjian, Hum Gene Ther, 5, 1371, (1994).
9. J. Rubin, J. Charboneau, C. Reading, and J. S. Kovach, Hum Gene Ther., 5, 1385 (1994).
10. N. J. Vogelzang, T. M. Lestingi, G. Sudakoff, and S. A. Kradjian, Hum Gene Ther., 5, 1357, (1994).
11. M. Grossman, S. E. Raper, K. Kozarsky, E. A. Stein, J. F. Engelhardt, D. Muller, P. J. Lupien, and J. M. Wilson, Nature Genetics, 6, 335 (1994).
12. M. S. Brown, J. L. Goldstein, R. J. Havel, D. Steinberg, Nature Genetics, 7, 349 (1994).
13. J. Zabner, L. A. Couture, R. J. Gregory, S. M. Graham, A. E. Smith, and M. J. Welsh, Cell, 75, 207 (1993).
14. R. G. Crystal, N. G. McElvaney, M. A. Rosenfeld, C-S Chu, A. Mastrangeli, J. G. Hay, S. L. Brody, H. A. Jaffe, N. Eissa, and C. Danel, Nature Genetics, 8 (1994).
15. N. G. McElvaney and R. G. Crystal, Nature Medicine, 1, 182 (1995).
16. N. Caplen, E. W. F. W. Alton, P. G. Middleton, J. R. Dorin, B. J. Stevenson, X. Gao, S. R. Durham, P. K. Jeffery, M. E. Hodson, C. Coutelle, L. Huang, D. J. Porteous, R. Williamson, and D. M. Geddes, Nature Medicine, 1, 39 (1995).
17. E. Sorscher, J. Logan, R. A. Frizzell, R. K. Lyrene, Z. Bebok, J. Y. Dong, M. D. Duvall, P. L. Felgner, S. Matalon, L. Walker, and B. J. Wiatrak, Hum Gene Ther, 5, 1259 (1994).
18. B. E. Huber, C. A. Richards, and E. A. Austin, Ann N Y Acad Sci, 716, 104 (1994).
19. K. W. Culver, Z. Ram, and S. Walbridge et. al., Science, 256, 1550 (1992).
20. Z. Ram, K. W. Culver, S. Walbridge, R. M. Blaese, and E. Oldfield, Cancer Res, 53, 83 (1993).
21. E. Oldfield, Z. Ram, K. W. Culver, R. M. Blaese, and H. L. DeVroom, Hum Gene Ther, 4, 39 (1993).
22. K. W. Culver, J. VanGilder, C. J. Link, T. Carlstrom, T. Buroker, W. Yuh, K. Koch, K. Schabold, S. Doornbas, B. Wetjen, and R. M. Blaese, Hum Gene Ther, 5, 343 (1994).

23. C. Raffel, K.W. Culver, D. Kohn, M. Nelson, S. Siegel, F. Gillis, C. J. Link, and J. G. Villablanca, Hum Gene Ther, 5, 863 (1994).
24. E. Oldfield, Z. Ram, Y. Chiang, and R. M. Blaese, Hum Gene Ther, 6, 55 (1995).
25. D. Bara, J. Hardin, M. Sadelain, and F. H. Gage, Proc Natl Acad Sci U S A, 91, 4348 (1994).
26. S-H Chen, H. D. Shine, J. C. Goodman. R. G. Grossman, S. L. C. Woo, Proc Natl Acad Sci U S A, 91, 3054 (1994).
27. M. J. Perez-Cruet, T. W. Trask, S-H Chen, J. C. Goodman, S.L.C. Woo, R. G. Grossman, and H.D. Shine, J Neuro Res, 39, 506 (1994).
28. C.A. Mullen, M.M. Coale, R. Lowe, and R. M. Blaese, Cancer Res, 54, 1503 (1994).
29. B. E. Huber, E. A. Austin, S. S. Good, V. C. Knick, S. Tibbeis, and C. A. Richards, Cancer Res, 53, 4619 (1993).
30. M. X. Wei, T. Tamiya, M. Chase, E. J. Boviatsis, T. K. H. Chang, N. W. Kowall, F. H. Hochberg, D. J. Waxman, X. O. Breakefield, and E. A. Chiocca, Hum Gene Ther, 5, 969 (1994).
31. E. G. Nabel, Circulation, 91, 541 (1995).
32. E. Nabel and G. Nabel, U S Patent 5,328,470 (1994).
33. G. Kolata, The N Y Times, C1, Dec 13, 1994.
34. R. Riessen, H. Rahimizadeh, E. Blessing, S. Takeshita, J. J. Barry, and J. M. Isner, Hum Gene Ther, 4, 749 (1993).
35. T. Ohno, D. Gordon, H. San, V. J. Pompilli, M. J. Imperiale, G. J. Nabel,E. G. Nabel, Science, 265, 781 (1994).
36. M. W. Chang, E. Barr, J. Seltzer, Y-Q Jiang, G. J. Nabel, E. G. Nabel, M. S. Parmacek, and J. M. Leiden, Science, 267, 518, (1995).
37. H. E. Von Der Leyen, G. H. Gibbons, R. Morishita, N. P. Lewis, L. Zhang, M. Nakajima, Y. Nakenda, J. P. Cooke, and V. J. Dzau, Proc. Natl. Acad. Sci, 92, 1137 (1995).
38. B. Dropulic and K-T Jeang, Hum Gene Ther, 5, 927 (1994).
39. C. Woffendin, Z-Y Yang, Udaykumar, L. Xu, N-S Yang, M. J. Sheehy, and G. J. Nabel, Proc. Natl. Acad. Sci, USA, 91, 11581 (1994).
40. G. J. Nabel, B.A. Fox, L. Post, C. B. Thompson, and C. Woffendin, Hum Gene Ther, 5, 79 (1993).
41. S. Suhr and F. H. Gage, Arch Neurol, 50, 1252 (1993).
42. T. Friedmann, TIG, 10, 210 (1994).
43. M. G. Kaplitt, P. Leone, R. J. Samulski, X. Xiao, D. W. Pfaff, K. L. O'Malley, and M. During, Nature Genetics, 8, 148 (1994).
44. G. Bandara, G. M. Mueller, J. Galea-Lauri, M. H. Tindal, H. I. Georgescu, M. K. Suchanek, G. L. Hung, J. C. Glorioso, P. D. Robbins, and C. H. Evans, Proc Natl Acad Sci U S A, 90, 10764 (1993).
45. G. L. Hung, J. Galea-Lauri, G. M. Mueller, H. I. Georgescu, L. A. Larking, M. K. Suchanek, M. H. Tindal, P. D. Robbins, and C. H. Evans, Gene Ther, 1, 64 (1994).
46. B. J. Roessler, J. W. Hartman, D.K. Vallance, J. M. Lata, S. L. Janich, B.L. Davidson, Hum Gene Ther, 6, 307 (1995).
47. R.M. Kotin, M. Siniscalco, R. J. Samulski, X. Zhu, L. Hunter, C. A. Laughlin, S. Mc Laughlin, N. Muzyczka, M. Rocchi, and K. I. Berns, Proc Natl Acad Sci U S A, 87, 2211 (1990).
48. S. D. Lupton, L. L. Brunton, V. A. Kalberg, and R. W. Overell, Mol Cel Biol, 11, 3374, (1991).
49. S. R. Riddell, P. D. Greenberg, R. W. Overell, T. P. Loughran, M. J. Gilvert, S. D. Lupton, J. Agosti, S. Scheeler, R. W. Coombs, and L. Corey, Hum Gene Ther, 3, 319 (1992).
50. W. French Anderson, G. J. McGarrity, and R. C. Moen, Hum Gene Ther, 4, 311 (1993).
51. A. H. Schulick, K. D. Newman, R. Virmani, and D. A. Dichek, Circulation, 91, 2407 (1994).

52. P.A. Furth, L. St. Onge, H. Böger, P. Gruss, M. Gossen, A. Kistner, H. Bujard, and L. Hennighausen, Proc Natl Acad Sci U S A, 91, 9302 (1994)
53. Y. Wang, B. W. O'Malley, Jr., S. Y. Tsai, and B. W. O'Malley, Proc Natl. Acad Sci U S A, 91, 8180 (1994).
54. D. M. Spencer, T. J. Wandless, S. L. Schreiber, and G. R. Crabtree, Science, 262, 1019 (1993).

Chapter 24. SH2 and SH3 Domains: Choreographers of Multiple Signaling Pathways

Martyn C. Botfield and Jeremy Green
ARIAD Pharmaceuticals, Cambridge, MA 02139

Introduction - Cellular signal transduction is a deceptively simple concept: information is gleaned from the external environment and the appropriate pre-programmed cellular response is executed. Not surprisingly, the molecular reality of these events belies the conceptual simplicity. Individual cells must respond correctly to dozens of simultaneous stimuli and integrate the multiple signal cascades. Rather than being a simple performance by one or two principals and a small chorus, reality within the cell is more akin to several intricate productions being performed on the same stage at the same time. To complicate matters further, many of the principal players are shared between the multiple productions and must flawlessly enter and exit the various performances without impairing the movement of the other participants. How then is chaos averted? To a large degree signal transduction pathways are choreographed by modular SH2 and SH3 domains which mediate highly specific protein:protein interactions. SH2 (Src homology 2) domains are modules of ~100 amino acids that specifically bind phosphotyrosine-containing proteins and peptides (1,2). SH3 (Src homology 3) domains are modules of ~60 amino acids that bind to proline-rich sequences (3,4). A list of selected therapeutic targets possessing SH2 or SH3 domains is given in Table 1. The design of specific antagonists to these domains holds the promise of targeted treatment of a broad range of pathologies.

TABLE 1. Selected list of SH2- and SH3-containing therapeutic targets and their associated pathologies.

PATHOLOGY	TARGET	DOMAIN STRUCTURE	REFERENCES
AIDS	Lyn	SH3-SH2-kinase	5
	Hck	SH3-SH2-kinase	5
Allergy and Asthma	Syk	SH2-SH2-kinase	6-8
	Lyn	SH3-SH2-kinase	9
Anemia	SH-PTP1	SH2-SH2--phosphatase	10,11
Autoimmune Disease	ZAP	SH2-SH2-kinase	12-14
Breast Cancer	Grb2	SH3-SH2-SH3	15,16
	Grb7	PH-SH2	17
	Src	SH3-SH2-kinase	18
Cancer	p85	SH3-SH2-SH2	19-21
	Shc	SH2	22
	Grb2	SH3-SH2-SH3	22-25
	Gap	SH2-SH3-SH2-PH-GAP	26
CML and ALL	Grb2	SH3-SH2-SH3	27
	CrkL	SH2-SH3-SH3	28
Erythroleukemias	Shc	SH2	29
Inflammatory Disease	STATs	DNA-binding-SH3-SH2	30-32
	p47-*phox*	SH3-SH3	33-35
	p67-*phox*	SH3-SH3	33-35
pre-B-cell Leukemia	Btk	PH-SH3-SH2-kinase	36
Myelodysplastic Syndrome	Tec	PH-SH3-SH2-kinase	37
Osteoporosis	Src	SH3-SH2-kinase	38,39

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

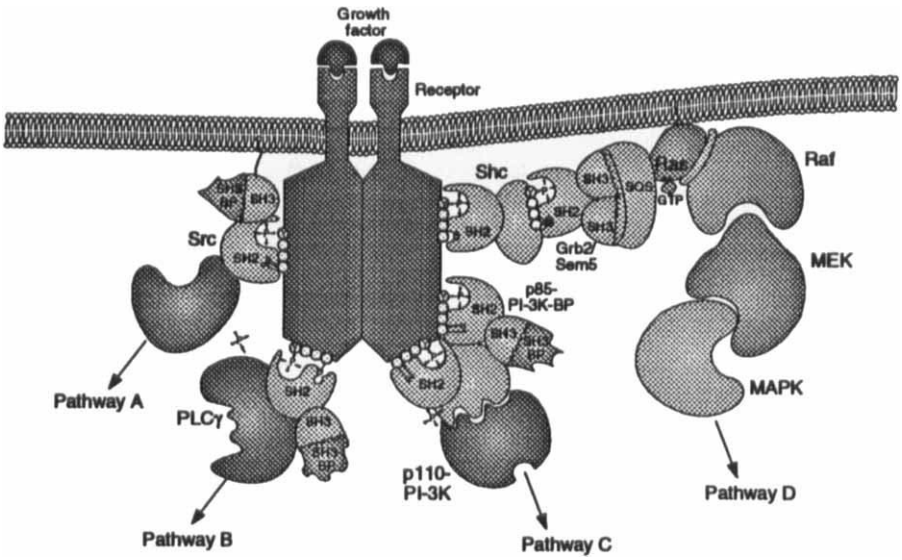


Figure 1 Schematized signal transduction pathways.

SIGNALING THROUGH MEMBRANE RECEPTORS

The role of SH2 and SH3 domains in signal transduction has been extensively reviewed (1,2,40-43). In growth factor, cytokine and antigen signaling, occupancy of a receptor by agonist results in receptor dimerization and the phosphorylation of regulatory tyrosines on the cytoplasmic surface (44). Phosphorylation is catalyzed by kinases that are a part of the receptor (receptor tyrosine kinases) or recruited to the receptor from the cytoplasm (non-receptor tyrosine kinases). The resulting phosphotyrosines permit binding of specific SH2-containing proteins and initiate a cascade of sequential protein interactions (Figure 1). Recruited molecules may themselves be tyrosine kinases and catalyze additional cycles of phosphorylation and SH2 recruitment (45); tyrosine phosphatases that terminate SH2-mediated associations (46,47); kinases or phosphatases that activate or inactivate previously bound enzymes (47); enzymes that generate lipid-derived second messengers (48); SH2/SH3 adapter proteins that act as docking stations for additional signaling molecules (49); or proteins that ultimately translocate to the nucleus and regulate transcription (30-32,50). Thus a single event -- extracellular binding of agonist -- can be coupled to a broad range of cellular responses through a series of SH2- and SH3-mediated events. These cellular responses include proliferation and differentiation (26,32,51-54), transcription (30-32,50), programmed cell death (55-57), adhesion, cytoskeletal rearrangement and chemotaxis (58,59), exo- and endocytosis (60,61), and assembly and activation of multi-subunit enzyme complexes (33-35).

SH2 DOMAINS

SH2 domains are small independently folded protein modules that bind to phosphotyrosine and permit phosphorylation dependent protein:protein interactions. The high affinity and specificity of the interaction permits a small numbers of molecules to survey and report the information state of the cell with a low risk of initiating false signals through random collision with other signaling molecules.

Structure - The three dimensional structure of a variety of ligated and unligated SH2 domains have been determined: src(62-64), Lck (65), PLCγ1 (66), Abl (67,68), PI3K (69), SH-PTP2 (70), and the tandem SH2 domains of ZAP-70 (71). Together they define a common structural fold, i.e. a central anti-parallel β-sheet bounded on either side by an α-helix. The two binding sites -- one on either side of the β-sheet -- are formed by close apposition of the helices to the sheet. The minimal phosphopeptide ligand is four amino acids (pYXXX) with a preference for a hydrophobic residue in the pY+3 position (72). Bound phosphopeptide is in an extended conformation that straddles the SH2 domain surface and is largely solvent exposed. The bulk of the interactions with the peptide involve phosphotyrosine and the pY+3 residue. Thus binding of phosphopeptide has been described as a "two pronged plug engaging a two-holed socket" (73). Only a small contribution is made by the pY+1 residue and little or no contact is made by the pY+2 residues or sites outside of the core motif. These structural predictions agree well with *in vitro* binding data (63,74). The phosphotyrosine binding cleft is formed by three strands of the β-sheet (βB, βC and βD), the loop between βB and βC (BC loop) and αA2 of the A helix (62-64). The βB strand is the site of the highly conserved FLVRES sequence (75). The central arginine of this sequence (Arg-178 in Src) is the only invariant residue and forms two hydrogen bonds with phosphate oxygens of the phosphotyrosine. Mutation of Arg-178 abolishes binding (74). It is likely that the position of this residue deep within the binding pocket places it beyond the reach of phosphoserine and phosphothreonine and thus explains the absence of demonstrable binding to peptides containing these residues. The hydrophobic pY+3 binding pocket is formed by two strands of the β-sheet (βD and βE) and the loops between βE/βF and αB/βG. The precise formation and side-chain composition of this pocket is more variable than that of the phosphotyrosine pocket and generates differences in both affinity and specificity (76,77). This is most notable in PLCγ1 and SH-PTP2 (Syp) where the pocket is reshaped to form an extended groove running across the ligand-binding surface (66,70). Molecular dynamic studies using nuclear magnetic resonance spectroscopy indicates that ligand binding induces little structural rearrangement other than side chain position within the binding clefts. Only the BC loop shifts towards a tighter packing with the phosphopeptide and protein core (78).

Affinity - The affinity of single SH2 domains for phosphoproteins and phosphopeptides derived from physiological targets range significantly. Using a variety of techniques, estimates from 10^{-5} to 10^{-9} M have been reported (72,76,79-81). Isothermal titration calorimetry suggests that the average change in standard free energy (ΔG^{o}) for the interaction between a single SH2 domain and a monophosphorylated ligand is approximately -8.5 kcal.mol^{-1} (81). Essentially no binding is detected when phosphotyrosine is replaced by tyrosine. Dissociation rates are very rapid (K_{diss}>0.1 sec^{-1} yielding $t_{1/2}$<0.2 minutes) for single SH2 domains (79,82,83). Tandem domains (SH2-SH2) have higher affinity ($\leq 10^{-9}$M) and slower dissociation rates (K_{diss} <1.5x10^{-4} sec^{-1} yielding $t_{1/2}$>100 minutes) for tandem binding sites suggesting that specificity, affinity and signal duration can all be amplified by the multimerization of binding domains. Tandem SH2 domains are found in a variety of proteins including Syk, ZAP-70, PLCγ, the p85 subunit of PI3K, SH-PTP1 and SH-PTP2.

Specificity - *In vitro* studies suggest that for isolated SH2 domains, the difference between the binding affinities of specific and non-specific phosphopeptides ranges from 10-1000 fold (63,76,83-85). Using degenerate phosphopeptide libraries, Cantley and colleagues determined the preferred binding sequences for Src, Fyn, Lck, Fgr, Abl, Grb2, Drk, Csk, Vav, SHC, fps/fes, Crk, Nck, Sem5, SH-PTP1, SH-PTP2, Syk, PLCγ1 and the p85 subunit of PI3K (72,86). The SH2 domains from p85 selected pYMXM or pYVXM, the exact sequences that have been identified as the p85-binding sites on the PDGF receptor, colony-stimulating factor 1 receptor and c-*kit*. All members of the Src family that where examined (Src, Fyn,

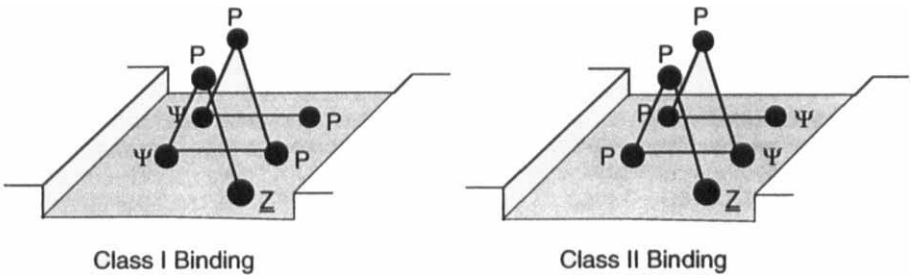


Figure 2 SH3 Class I (ZPΨPPΨP) *versus* Class II (ΨPPΨPPZ) ligand binding. P = Proline, Ψ = hydrophobic amino acid, Z = SH3-specific amino acid. See text for complete description.

Lyn, Lck and Fgr) selected pYEEI and defined a general consensus of pYEEΨ, where Ψ represents hydrophobic residues. Other members exhibited varying degrees of preference, usually with stronger selection for residues at the pY+3 and less selection at pY+1 and Y+2, *in concurrence with prediction from structure.* An exception to this rule were the SH2 domains from Sem5/Grb2/Drk which exhibited a marked preference for Asn at the pY+2 position and less preference at the pY+1 and pY+3 pockets. It is anticipated that expanding the set of structural monomers beyond the 20 naturally occurring amino acids will permit the synthesis of exquisitely selective SH2 antagonists.

SH3 DOMAINS

SH3 (Src homology 3) domains are independently folded protein modules of 55-70 amino acids that selectively bind proline-rich protein sequences (3,4). Although generally of lower affinity than SH2-mediated interactions, SH3 interactions are essential for multiple signaling cascades.

Structure - A variety of ligated and unligated SH3 structures have been reported including Src (87-89), PI3K (90-92), PLCγ (93), GAP (94), Fyn (95), Csk (96), Lck (65), Abl (97), GRB2 (98) and α–spectrin (88). Although there is variability, the basic fold is composed of eight β–strands arranged in two, three-member anti-parallel β-sheets (βI and βII) that form a compact core and a third, two-strand β-sheet (βIII; ref. 98). The binding "platform" is formed by residues of βII and βIII plus two negatively-charged hypervariable loops and is subdivided into three binding sites: a polar pocket and two hydrophobic pockets. Details of ligand binding are discussed below.

Class I *vs.* Class II Binding - Peptides bind as left-handed polyproline type II helices (89) in one of two orientations (Figure 2). Class I peptides bind with their amino-termini making contact with the polar pocket and carboxy-terminal residues intercalating the hydrophobic pockets. Class II peptides bind in the reverse orientation, i.e. with the carboxy-terminus making contact with the polar pocket and amino-terminal residues intercalating the hydrophobic sites. In both cases the same binding sites are occupied; only the orientation is reversed (89). Class I *versus* Class II orientation can be deduced directly from the peptide sequence (99). Class I ligands have the general form ZPΨPPΨP and Class II ligands ΨPPΨPPZ where Ψ is any hydrophobic amino acid and Z is an SH3-domain-specific residue (commonly arginine). When oriented in the binding platform, Z makes contact with the polar binding pocket and the two Ψ-Pro motifs occupy the two hydrophobic pockets. The proline residues that connect the three submotifs, i.e. ZPΨPPΨP (Class I) and ΨPPΨPPZ (Class II), form the third, non-contacting ridge of the proline helix and stabilize helix integrity. Class I *versus* Class II orientation is dictated by several features: the position of the Z residue relative to

the ΨP motifs; the position of the γ and δ methylenes of proline within the pocket; and the uniquely compact nature of the Ψ-Pro dipeptide. In a Ψ-Pro sequence, the C_α-C_β bond of Ψ is separated from the N-C_δ bond of proline by two backbone bonds; in a Pro-Ψ sequence, this separation is three backbone bonds. Thus a Pro-Ψ sequence has a composite hydrophobic surface that is significantly larger than that of an Ψ-Pro sequence and can not be accommodated by the binding site (89). Details of these features and their precise role in determining orientation are reviewed elsewhere (89,99,100).

Affinity - Estimates of the affinity of single SH3 domains for intact proteins or peptides derived from physiological targets range from $>10^{-3}$ to 10^{-7}M (25,89,92,98, 101-104) and are thus generally weaker than SH2/phosphotyrosine interactions. These modest affinities can be enhanced by multimerization. For example, the affinity of the adapter protein Grb2 (SH3-SH2-SH3) has a K_D~25 nM for a substrate that permits simultaneous interaction with both SH3 domains (25). No inherent difference in Class I *versus* Class II affinity has been reported.

Specificity - SH3 specificity has been explored using phage display (102,105,106) and random peptide libraries (92,101). Core motif peptides are relatively non-selective and bind to a broad range of SH3 domains. For example, the optimum Class I core for Src is RPLPPLP (102,105,106). This sequence also binds to the SH3 domains of Lyn, Fyn, Yes, and PI3K (102). It does not, however, bind to the Abl SH3 domain (102). This selectivity is imparted by the amino-terminal arginine and marks the first determinant of specificity, i.e. the first (ZPΨPPΨP) or last (ΨPPΨPPZ) residue of Class I or Class II cores, respectively. SH3 domains with an aspartic or glutamic acid in a conserved position of the polar binding pocket (Asp-99 in Src) all bind peptides where Z is arginine. SH3 domains that have a non-acidic amino acid at this position, e.g. Abl, fail to bind arginine peptides and select for alternative residues. Additional specificity is derived from as many as five residues either side of the core, i.e. XXXXZPYPPYPXXXX or XXXXΨPPΨPPZXXXX (102). Flanking sequences specific for the SH3 domains of Src, Fyn, Lyn, Yes PI3K and Abl have all been reported (102).

INVOLVEMENT OF SH2 AND SH3 DOMAINS IN DISEASE

Human pathologies correlated with *dysfunction* of SH2 and SH3 domains are listed in Table 2. The precise molecular contributions of these domains in precipitating disease have been identified in several cases. The following examples are chosen to illustrate both the complexity of the cellular signaling pathways and the diversity of roles fulfilled by SH2 and SH3 domains.

Chronic Myelogenous Leukemia (CML) and Acute Lymphoblastic Leukemia (ALL) - A reciprocal translocation between chromosomes 9 and 22 (the so called "Philadelphia chromosome") has been identified in many cases of CML and ALL (Ph+ CML and Ph+ ALL). This translocation results in the juxtaposition of the *bcr* gene and the *abl* proto-oncogene (107,116) and results in expression of 185 kDa or 210 kDa chimeric Bcr-Abl proteins correlated with ALL and CML, respectively (117,118). In both cases, the first 26 amino acids of the proto-oncogene c-Abl are replaced by the first 426 or 927 (or 902) amino acids of Bcr (107,117,118). The resulting chimeras localize to the cytoplasm and contain the SH3, SH2 and tyrosine kinase domains of c-abl. The presence of the Bcr sequence deregulates the kinase, an essential requirement for transformation (119,120). Bcr-Abl forms a physical complex with a variety of signaling molecules including Grb2 (27,110), SH-PTP2 (121), and CrkL (122). Grb2 is a 25 kDa adapter protein of the form SH3-SH2-SH3 sequence. The SH2 domain of Grb2 recognizes and binds to the *bcr*-encoded Y177 tyrosine autophosphorylation site in both the p185 and p210 Bcr-Abl proteins. Grb2 in turn associates with mSOS (a Ras guanine nucleotide exchange protein) through a Grb2-SH3/mSOS-poly-proline interaction and

TABLE 2 Selected human pathologies correlated with dysfunction of SH2- and SH3-containing proteins.

PATHOLOGY	PROTEIN	DOMAIN STRUCTURE	REFERENCES
Chronic Myelogenous Leukemia	Bcr/Abl	SH3-SH2-kinase	107-109
	Grb2	SH3-SH2-SH3	27,110
	CrkL	SH2-SH3-SH3	28
X-Linked Agammaglobulinemia	Btk	PH-SH3-SH2-kinase	111,112
Myelodysplastic Syndrome	Tec	PH-SH3-SH2-kinase	37
Chronic Granulomatous Disease	p47-*phox*	SH3-SH3	35
	p67-*phox*	SH3-SH3	35
Severe Combined Immunodeficiency	ZAP-70	SH2-SH2-kinase	113,114
Faciogenital Dysplasia	FGD1	SH3-binding site	115

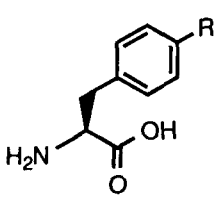
(activates the Ras pathway leading to deregulated mitogenic signals (27,110). Mutation of Y177 to phenylalanine (Y177F) abolishes GRB-2 binding and abrogates Bcr-Abl-induced Ras activation and transformation (27). Conversely, point mutations that severely impair the ability of the SH2 domain to bind phosphotyrosine R552L in p185) or removes the major tyrosine autophosphorylation in the kinase domain (Y813F in p185), also impair transformation by Bcr-Abl without effecting Grb2 binding (123). These results suggest that Grb2 binding is necessary, but not sufficient for transformation. The second factor may be CrkL, a 39 kDa adapter protein of the form SH2-SH3-SH3. In neutrophils from normal patients, CrkL is unphosphorylated. In neutrophils from Ph+ CML patients (28,124) or *bcr-abl* transformed cell lines (122), CrkL is highly and constitutively tyrosine phosphorylated. Treatment of normal neutrophils with a variety of cytokines and agonists fails to induce CrkL phosphorylation suggesting that this is not part of a normal signaling pathway (124). Furthermore, CrkL forms a physical complex with Bcr-Abl in transformed cells and is readily tyrosine phosphorylated by the Bcr-Abl and c-Abl kinases *in vitro* (122). Together these results implicate CrkL as a second potential oncogenic mediator of Bcr-Abl.

Chronic Granulomatous Disease (CGD) - CGD is characterized by a failure of neutrophils to generate microbicidal oxidants (e.g. superoxide) and leaves CGD patients highly susceptible to opportunistic infections (35). The enzyme responsible, NADPH oxidase, is composed of four proteins: p22-*phox*, p47-*phox*, p67-*phox* and gp91-*phox*. Activation of NADPH oxidase is regulated through assembly, i.e. upon stimulation of the phagocyte, p47-*phox* and p67-*phox* are translocated from the cytosol to the membrane where they associate with the membrane-bound p22-*phox*/gp91-*phox*/heme complex and activate superoxide generation. This process relies on a series of SH3-mediated events that begins with the dissociation of an intramolecular proline-rich binding sequence from the p47-*phox* SH3-domain that unmasks the SH3 domain (34). Following unmasking, both the SH3 and proline-rich sequences become available to participate in other *inter*molecular interactions. Unmasked p47-*phox* then associates with p67-*phox* through SH3-mediated interactions to form a p47/p67 heterodimer (34) which in turn associates with the membrane-bound components through one or both SH3 domains of p47-*phox* and proline-rich sequences of p22-*phox* (34). Mutation of Pro-156 to glutamine in the p22-*phox* disrupts one proline-rich sequence and abolishes binding (33,34). An identical mutation has been found in a patient with CGD (125), suggesting that it represents a physiological binding sequence. Synthetic peptides corresponding to proline-rich SH3-binding sites of p22-*phox* or p47-*phox* effectively block *in vitro* oxidase assembly and activation (33,34,126).

THE CHALLENGES FOR DRUG DESIGN

The design of SH2 inhibitors presents novel challenges. Paramount among these is cell permeability and phosphatase resistance. Although few compounds have been reported in the literature, several phosphatase resistant mimics and cell-permeable phosphate "pro-drugs" deserve note.

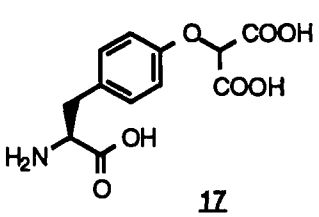
Phosphotyrosine Mimics - Phosphotyrosine (1) can most easily be rendered resistant to phosphatases by replacement of the tyrosine oxygen with a CH_2 as in 5. Numerous syntheses, both racemic (127) and asymmetric (128), have been reported. Peptides containing 5 as a replacement for phosphotyrosine show a minimum 6-fold weaker binding depending on the SH2 domain examined (63,129,130). Crystal structures of the Lck SH2 domain in complex with two separate peptides prepared with 5 have been solved (131). The conformation of the SH2 domain is largely unchanged from the phosphotyrosine complex, with the notable exception of a perturbation in the BC loop ("phosphotyrosine-binding" loop) that connects strands βB and βC. The observed position of the BC loop suggests the loss or absence of up to three hydrogen bonds between the loop and the phosphonate. It has been rationalized that the decreased affinity of this analog may be a result of either the lower acidity of this residue (pK_a2 = 7.1) relative to phosphotyrosine (pK_a2 = 5.7), or the loss of hydrogen bonding between the phosphate oxygen SH2 domain, or a combination of both (130). To test these hypotheses, peptides were prepared with phosphonate analogs bearing electron withdrawing substituents α to phosphorus in order to increase the acidity of the phosphonate. Inclusion of a single fluorine atom (4) increased affinity 2-fold, while a second fluorine (2) re-established a level of binding equal to phosphotyrosine (1). The utility of peptides prepared with 2 has been demonstrated in T-cells. Peptides corresponding to the third immunoreceptor tyrosine-based activation motif (ITAM) of the T-cell receptor ζ-chain were able to selectively inhibit the association of the T-cell receptor with the tyrosine kinase ZAP-70 in permeabilized T-cells (14). 2-containing peptides also effectively block SH-PTP2 mediated mitogenic signaling in rat fibroblasts (132). In both studies, the equivalent phosphotyrosine peptides showed no efficacy, presumably due to removal of the labile phosphate via cellular phosphatases.



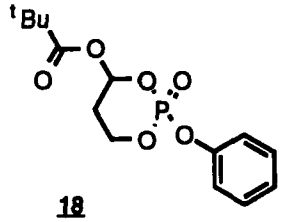
		relative affinity
1	R = OPO_3H_2	1
2	R = $CF_2PO_3H_2$	1
3	R = $OPSO_2H_2$	2
4	R = $CHFPO_3H_2$	3
5	R = $CH_2PO_3H_2$	6
6	R = $CHOHPO_3H_2$	20
7	R = $CHOHPO_3H_2$	25
8	R = $CH_2PO_2H_2$	40
9	R = CH_2SO_3H	300
10	R = CH_2CH_2COCHO	370
11	R = $CH_2CH_2COCOCH_3$	520
12	R = CH_2COOH	940
13	R = $CH_2CONHOH$	>1000
14	R = NH_2	>1000
15	R = NO_2	>1000
16	R = OH	>1000

In extensive studies of the binding preferences of the Src SH2 domain for peptide-based ligands (63,133,134), a number of potential phosphotyrosine mimics were assayed against Src SH3-SH2 with variable effectiveness (see table associated with 1-16). Although essentially inactive, compounds 10 and 11 represent a novel

attempt to "trap" the arginine residues that participate in phosphotyrosine binding. Several non-phosphorus mimics (9-17) were also examined with 17 being the only candidate exhibiting activity (135). Although peptides incorporating 17 showed no affinity for Src and Grb2, peptides specific for PI3K showed measurable, albeit 100-fold reduced, affinity for the p85 SH2 domains (63). Furthermore, binding of 17 to the N-terminal SH2 of the phosphatase SH-PTP2 was equal to that observed with the corresponding 6-containing peptide. Cellular studies using peptides prepared with 17 will be valuable in assessing the potentials of this promising phosphotyrosine mimic.



17



18

Phosphate/Phosphonate Pro-drugs - Should the challenge of creating a cell permeable phosphotyrosine mimic prove elusive, the alternative strategy of neutralizing the dianionic phosphate in the form of a pro-drug may be viable. The use of phosphate diesters have previously been applied in other areas where masking of phosphate is required, such as nucleotide anti-virals (136). One limitation of the diester protecting group approach is the reduced susceptibility of the anionic protecting group monoester to hydrolytic enzymes following cleavage of the first ester group from the neutral molecule. This can be circumvented by removing the site of hydrolysis far from the phosphorus atom, such as in the application of di(4-acetoxybenzyl) phosphonates (137). A recent novel approach is presented in a model "masked" phosphate, 18. Through the action of cellular esterases, the labile pivaloyl ester is converted to an unstable hemiacetal phosphate triester, which decomposes to reveal the phosphate (138). It will be interesting to see how these and others compounds evolve.

References

1. T. Pawson, Nature 373, 573 (1995).
2. G.B. Cohen, R. Ren and D. Baltimore, Cell 80, 237 (1995).
3. A. Musacchio, M. Wilmanns and M. Saraste, Prog. Biophys. Mol. Biol. 61, 283 (1994).
4. C.J. Morton and I.D. Campbell, Curr. Biol. 4, 615 (1994).
5. K. Saksela, G. Cheng and D. Baltimore, EMBO J. 14, 484 (1995).
6. D.L. Durden and Y.B. Liu, Blood 14, 2102 (1994).
7. J. Oliver, D.L. Burg, B.S. Wilson, J.L. McLaughlin and R.L. Gaehlen, J. Biol. Chem. 269, 29697 (1994).
8. L. Shiue, J. Green, O.M. Green, J.L. Karas, J.P. Morgenstern, M.K. Ram, M.K. Taylor, M.J. Zoller, L.D. Zydowsky, J.B. Bolen and a.l. et, Mol. Cell. Biol. 15, 272 (1995).
9. T. Yamashita, S.-Y. Mao and H. Metzger, Proc. Natl. Acad. Sci. USA 91, 11251 (1994).
10. T. Yi, J. Zhang, O. Miura and J.N. Ihle, Blood 85, 87 (1995).
11. J.N. Ihle, B.A. Witthuhn, F.W. Quelle, O. Silvennoinen, B. Tang and T. Yi, Blood Cells 20, 65 (1994).
12. T. Mustelin, Immunity 1, 351 (1994).
13. N. Ishii, H. Asao, Y. Kimura, T. Takeshita, M. Nakamura, S. Tsuchiya, T. Konno, M. Maeda, T. Uchiyama and K. Sugamura, J. Immunol. 153, 1310 (1994).
14. R.L. Wange, N. Isakov, T.J. Burke, A. Otaka, P.P. Roller, J.D. Watts, R. Aebersold and L.E. Samelson, J. Biol. Chem. 270, 944 (1995).
15. P.W. Janes, R.J. Daly, A. deFazio and R.L. Sutherland, Oncogene 9, 3601 (1994).
16. R.J. Daly, M.D. Binder and R.L. Sutherland, Oncogene 9, 2723 (1994).

17. D. Stein, J. Wu, S.A. Fuqua, C. Roonprapunt, V. Yajnik, P. D'Eustachio, J.J. Moskow, A.M. Buchberg, C.K. Osborne and B. Margolis, Embo J. 13, 1331 (1994).
18. D.K. Luttrell, A. Lee, T.J. Lansing, R.M. Crosby, K.D. Jung, D. Willard, M. Luther, M. Rodriguez, J. Berman and T.M. Gilmer, Proc. Natl. Acad. Sci. U S A 91, 83 (1994).
19. Q. Hu, A. Klippel, A.J. Muslin, W.J. Fantl and L.T. Williams, Science 268, 100 (1995).
20. B.H. Jhun, D.W. Rose, B.L. Seely, L. Rameh, L. Cantley, A.R. Saltiel and J.M. Olefsky, Mol. Cell. Biol. 14, 7466 (1994).
21. M.J. Fry and M.D. Waterfield, Philos. Trans. R. Soc. Lond. B Biol. Sci. 340, 337 (1993).
22. K.S. Ravichandran, U. Lorenz, S.E. Shoelson and S.J. Burakoff, Mol. Cell. Biol. 15, 593 (1995).
23. M.M. Gottesman, J. Natl. Cancer. Inst. 86, 1277 (1994).
24. P. Chardin, J.H. Camonis, N.W. Gale, A.L. van, J. Schlessinger, M.H. Wigler and S.D. Bar, Science 260, 1338 (1993).
25. N. Li, A. Batzer, R. Daly, V. Yajnik, E. Skolnik, P. Chardin, S.D. Bar, B. Margolis and J. Schlessinger, Nature 363, 85 (1993).
26. S.A. Moodie and A. Wolfman, Trends Genet. 10, 44 (1994).
27. A.M. Pendergast, L.A. Quilliam, L.D. Cripe, C.H. Bassing, Z. Dai, N. Li, A. Batzer, K.M. Rabun, C.J. Der, J. Schlessinger and a.l. et, Cell 75, 175 (1993).
28. T. Oda, C. Heaney, J.R. Hagopian, K. Okuda, J.D. Griffin and B.J. Druker, J. Biol. Chem. 269, 22925 (1994).
29. D.L. Barber, J.C. DeMartino, M.O. Showers and A.D. D'Andrea, Mol. Cell. Biol. 14, 2257 (1994).
30. J.N. Ihle, Proc. Soc. Exp. Biol. Med. 206, 268 (1994).
31. J.E. Darnell Jr., I.M. Kerr and G. Stark, Science 264, 1415 (1994).
32. J.N. Ihle, B.A. Wittuhn, F.W. Quelle, K. Yamamoto, W.E. Thierfelder, B. Kreider and O. Silvennoinen, TIBS 19, (1994).
33. T.L. Leto, A.G. Adams and M.I. de, Proc. Natl. Acad. Sci. U S A 91, 10650 (1994).
34. H. Sumimoto, Y. Kage, H. Nunoi, H. Sasaki, T. Nose, Y. Fukumaki, M. Ohno, S. Minakami and K. Takeshige, Proc. Natl. Acad. Sci. U S A 91, 5345 (1994).
35. L.C. McPhail, J. Exp. Med. 180, 2011 (1994).
36. M. de Weers, R.G. Mensink, M.E. Kraakman, R.K. Schuurman and R.W. Hendriks, Hum. Mol. Genet. 3, 161 (1994).
37. K. Sato, H. Mano, T. Ariyama, J. Inazawa, Y. Yazaki and H. Hirai, Leukemia 8, 1663 (1994).
38. K.B. Kaplan, K.B. Bibbins, J.R. Swedlow, M. Arnaud, D.O. Morgan and H.E. Varmus, Embo J. 13, 4745 (1994).
39. C. Lowe, T. Yoneda, B.F. Boyce, H. Chen, G.R. Mundy and P. Soriano, Proc. Natl. Acad. Sci. USA 90, (1993).
40. J. Schlessinger, Curr. Opin. Genet. Dev. 4, 25 (1994).
41. B.J. Mayer and D. Baltimore, Trends Cell Biol. 3, 8 (1993).
42. G. Panayotou and M.D. Waterfield, Bioessays 15, 171 (1993).
43. G. Carpenter, FASEB J. 6, 3283 (1992).
44. C.-H. Heldin, Cell 80, 213 (1995).
45. J.C. Cambier and W.A. Jensen, Curr. Opin. Genet. Dev. 4, 55 (1994).
46. G.S. Feng and T. Pawson, Trends Genet. 10, 54 (1994).
47. T. Hunter, Cell 80, 225 (1995).
48. N. Divecha and R.F. Irvine, Cell 80, 269 (1995).
49. J. Downward, FEBS Lett. 338, 113 (1994).
50. C.S. Hill and R. Treisman, Cell 80, 199 (1995).
51. D.R. Edwards, Trends Pharm. Sci. 15, 239 (1994).
52. G.C. Prendergast and J.B. Gibbs, Bioessays 16, 187 (1994).
53. M.G.J. Myers, X.J. Sun and M.F. White, TIBS 19, 289 (1994).
54. J. Blenis, Proc. Natl. Acad. Sci. USA 90, 5889 (1993).
55. D.R. Green and D.W. Scott, Curr. Op. Immunol. 6, 476 (1994).
56. E.A. Harrington, A. Fanidi and G.I. Evan, Curr. Op. Genet. Dev. 4, (1994).
57. M.K. Collins, G.R. Perkins, G. Rodriguez-Tarduchy, M.A. Nieto and A. Lopez-Rivas, Bioessays 16, 133 (1994).
58. E.A. Clark and J.S. Brugge, Science 268, 233 (1995).
59. A. Bretscher, Curr. Opin. Cell. Biol. 5, 653 (1993).
60. P.S. McPherson, A.J. Czernik, T.J. Chilcote, F. Onofri, F. Benfenati, P. Greengard, J. Schlessinger and C.P. De, Proc. Natl. Acad. Sci. U S A 91, 6486 (1994).
61. H. Miki, K. Miura, K. Matuoka, T. Nakata, N. Hirokawa, S. Orita, K. Kaibuchi, Y. Takai and T. Takenawa, J. Biol. Chem. 269, 5489 (1994).
62. G. Waksman, S.E. Shoelson, N. Pant, D. Cowburn and J. Kuriyan, Cell 72, 779 (1993).
63. T. Gilmer, M. Rodriguez, S. Jordan, R. Crosby, K. Alligood, M. Green, C. Wagner, D. Kinder, P. Charifson, A. Hassel, D. Willard, M. Luther, D. Rusnal, D. Stembach, M. Mehrotha, M. Peel, L. Shampine, R. Davis, J. Robins, I. Patel, D. Kassel, W. Burkhart, M. Moyer, T. Bradshaw and J. Berman, J. Biol. Chem. 269, 31711 (1994).

64. R.X. Xu, J.M. Word, D.D. G., M.J. Rink, D.H. Willard Jr and R.T. Gampe Jr, Biochem. 34, 2107 (1995).
65. M.J. Eck, S.K. Atwell, S.E. Shoelson and S.C. Harrison, Nature 368, 764 (1994).
66. S.M. Pascal, A.U. Singer, G. Gish, T. Yamazaki, S.E. Shoelson, T. Pawson, L.E. Kay and K.J. Forman, Cell 77, 461 (1994).
67. M. Overduin, C.B. Rios, B.J. Mayer, D. Baltimore and D. Cowburn, Cell 70, 697 (1992).
68. M. Overduin, B. Mayer, C.B. Rios, D. Baltimore and D. Cowburn, Proc. Natl. Acad. Sci. U S A 89, 11673 (1992).
69. G.W. Booker, A.L. Breeze, A.K. Downing, G. Panayotou, I. Gout, M.D. Waterfield and I.D. Campbell, Nature 358, 684 (1992).
70. C.H. Lee, D. Kominos, S. Jacques, B. Margolis, J. Schlessinger, S.E. Shoelson and J. Kuriyan, Structure 2, 423 (1994).
71. M. Hatada, X. Lu, E.R. Laird, J. Karas and M. Zoller, Nature *in press*, (1995).
72. Z. Songyang, S.E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Haser, F. King, T. Roberts, S. Ratnofsky, R.J. Lechleider, B.G. Neel, R.B. Birge, J.E. Fajardo, M.M. Chou, H. Hanafusa, B. Schaffhausen, and L.C.Cantley, Cell 72, 767 (1993).
73. H. Yu and S.L. Schreiber, unpublished, (1994).
74. K.B. Bibbins, H. Boeuf and H.E. Varmus, Mol. Cell. Biol. 13, 7278 (1993).
75. M. Hidaka, Y. Homma and T. Takenawa, Biochem. Biophys. Res. Commun. 180, 1490 (1991).
76. L.E. Marengere, Z. Songyang, G.D. Gish, M.D. Schaller, J.T. Parsons, M.J. Stern, L.C. Cantley and T. Pawson, Nature 369, 502 (1994).
77. A. von Bonin, J. Wienands, U. Manning, J.F. Zuber and G. Baumann, J. Biol. Chem. 269, 33035 (1994).
78. N.A. Farrow, R. Muhandiram, A.U. Singer, S.M. Pascal, C.M. Kay, G. Gish, S.E. Shoelson, T. Pawson, K.J. Forman and L.E. Kay, Biochem. 33, 5984 (1994).
79. G. Zhu, S.J. Decker, B.J. Mayer and A.R. Saltiel, J Biol Chem 268, 1775 (1993).
80. G. Payne, S.E. Shoelson, G.D. Gish, T. Pawson and C.T. Walsh, Proc. Natl. Acad. Sci. U S A 90, 4902 (1993).
81. J.E. Ladbury, M.A. Lemmon, M. Zhou, J. Green, M.C. Botfield and J. Schlessinger, Proc. Natl. Acad. Sci. USA 92, 3199 (1995).
82. G. Panayotou, G. Gish, P. End, O. Truong, I. Gout, R. Dhand, M.J. Fry, I. Hiles, T. Pawson and M.D. Waterfield, Mol. Cell. Biol. 13, 3567 (1993).
83. S. Felder, M. Zhou, P. Hu, J. Urena, A. Ullrich, M. Chaudhuri, M. White, S.E. Shoelson and J. Schlessinger, Mol. Cell. Biol. 13, 1449 (1993).
84. E. Piccione, R.D. Case, S.M. Domchek, P. Hu, M. Chaudhuri, J.M. Backer, J. Schlessinger and S.E. Shoelson, Biochem. 32, 3197 (1993).
85. G. Payne, L.A. Stolz, D. Pei, H. Band, S.E. Shoelson and C.T. Walsh, Chem. Biol. 1, 99 (1994).
86. Z. Songyang, S.E. Shoelson, J. McGlade, P. Olivier, T. Pawson, X.R. Bustelo, M. Barbacid, H. Sabe, H. Hanafusa, T. Yi and a.l. et, Mol. Cell. Biol. 14, 2777 (1994).
87. H. Yu, M.K. Rosen, T.B. Shin, D.C. Seidel, J.S. Brugge and S.L. Schreiber, Science 258, 1665 (1992).
88. A. Musacchio, M. Noble, R. Pauptit, R. Wierenga and M. Saraste, Nature 359, 851 (1992).
89. S. Feng, J.K. Chen, H. Yu, J.A. Simon and S.L. Schreiber, Science 266, 1241 (1994).
90. S. Koyama, H. Yu, D.C. Dalgarno, T.B. Shin, L.D. Zydowsky and S.L. Schreiber, FEBS Lett. 324, 93 (1993).
91. S. Koyama, H. Yu, D.C. Dalgarno, T.B. Shin, L.D. Zydowsky and S.L. Schreiber, Cell 72, 945 (1993).
92. H. Yu, J.K. Chen, S. Feng, D.C. Dalgarno, A.W. Brauer and S.L. Schreiber, Cell 76, 933 (1994).
93. D. Kohda, H. Hatanaka, M. Odaka, V. Mandiyan, A. Ullrich, J. Schlessinger and F. Inagaki, Cell 72, 953 (1993).
94. Y.S. Yang, C. Garbay, M. Duchesne, F. Cornille, N. Jullian, N. Fromage, B. Tocque and B.P. Roques, EMBO J. 13, 1270 (1994).
95. M.E. Noble, A. Musacchio, M. Saraste, S.A. Courtneidge and R.K. Wierenga, EMBO J. 12, 2617 (1993).
96. T.V. Borchert, M. Mathieu, J.P. Zeelen, S.A. Courtneidge and R.K. Wierenga, FEBS Lett. 341, 79 (1994).
97. A. Musacchio, M. Saraste and M. Wilmanns, Nat. Struct. Biol. 1, 546 (1994).
98. D. Kohda, H. Terasawa, S. Ichikawa, K. Ogura, H. Hatanaka, V. Mandiyan, A. Ullrich, J. Sclessinger and F. Inagaki, Curr. Biol. 2, 1029 (1994).
99. J. Simon and S.L. Schreiber, Chem. Biol. 2, 53 (1995).
100. W.A. Lim, F.M. Richards and R.O. Fox, Nature 372, 375 (1994).
101. J.K. Chen, W.S. Lane, A. Brauer, A. Tanaka and S.L. Schreiber, J. Am. Chem. Soc. 115, 12591 (1993).
102. R.J. Rickles, M.C. Botfield, Z. Weng, J.A. Taylor, O.M. Green, J.S. Brugge and M.J. Zoller, EMBO J. 13, 5598 (1994).

103. W.A. Lim, R.O. Fox and F.M. Richards, Protein Sci. 3, 1261 (1994).
104. R.J. Rickles, M.C. Botfield, X. Zhou, P.A. Henry, J. Brugge and M.J. Zoller, Proc. Natl. Acad. Sci. USA *in press*, (1995).
105. C. Cheadle, Y. Ivashchenko, V. South, G.H. Searfoss, S. French, R. Howk, G.A. Ricca and M. Jaye, J. Biol. Chem. 269, 24034 (1994).
106. A.B. Sparks, L.A. Quilliam, J.M. Thorn, C.J. Der and B.K. Kay, J. Biol. Chem. 269, 23853 (1994).
107. E. Shtivelman, B. Lifschitz, R.P. Gale and E. Canaani, Nature 315, 550 (1985).
108. B. Clarkson, A. Strife, A. Perez, C. Lambek and D. Wisniewski, Leuk. Lymphoma 2, 81 (1993).
109. A. Serra, A. Guerrasio, G. Gaidano, C. Rosso, C.G. Rege, D. Petroni, U. Mazza and G. Saglio, Leuk. Lymphoma 1, 25 (1993).
110. L. Puil, J. Liu, G. Gish, G. Mbamalu, D. Bowtell, P.G. Pelicci, R. Arlinghaus and T. Pawson, EMBO J. 13, 764 (1994).
111. Q. Zhu, M. Zhang, D.J. Rawlings, M. Vihinen, T. Hagemann, D.C. Saffran, S.P. Kwan, L. Nilsson, C.I. Smith, O.N. Witte and a.l. et, J. Exp. Med. 180, 461 (1994).
112. M. Vihinen, L. Nilsson and C.I. Smith, Biochem. Biophys. Res. Commun. 205, 1270 (1994).
113. A.C. Chan, T.A. Kadlecek, M.E. Elder, A.H. Filipovich, W.L. Kuo, M. Iwashima, T.G. Parslow and A. Weiss, Science 264, 1599 (1994).
114. S.D. Voss, R. Hong and P.M. Sondel, Blood 83, 626 (1994).
115. N.G. Pasteris, A. Cadle, L.J. Logie, M.E. Porteous, C.E. Schwartz, R.E. Stevenson, T.W. Glover, R.S. Wilroy and J.L. Gorski, Cell 79, 669 (1994).
116. S. Iwata, S. Mizutani, S. Nakazawa and J. Yata, Leukemia 8, 1696 (1994).
117. L.C. Chan, K.K. Karhi, S.I. Rayter, N. Heisterkamp, S. Eridani, R. Powles, S.D. Lawler, J. Groffen, J.G. Foulkes, M.F. Greaves and L.M. Wiedemann, Nature 325, 635 (1987).
118. S.S. Clark, J. McLaughlin, W.M. Christ, R. Champlin and O.N. Witte, Science 235, 85 (1987).
119. A.M. Pendergast, A.J. Muller, M.H. Havlik, Y. Maru and O.N. Witte, Cell 66, 161 (1991).
120. A.J. Muller, J.C. Young, A.M. Pendergast, M. Pondel, N.R. Landau, D.R. Littman and O.N. Witte, Mol. Cell. Biol. 11, 1785 (1991).
121. T. Tauchi, G.S. Feng, R. Shen, H.Y. Song, D. Donner, T. Pawson and H.E. Broxmeyer, J. Biol. Chem. 269, 15381 (1994).
122. J. ten Hoeve, V. Kaartinen, T. Fioretos, L. Haataja, J.W. Voncken, N. Heisterkamp and J. Groffen, Cancer Res. 54, 2563 (1994).
123. D.E. Afar, A. Goga, J. McLaughlin, O.N. Witte and C.L. Sawyers, Science 264, 424 (1994).
124. G.L. Nichols, M.A. Raines, J.C. Vera, L. Lacomis, P. Tempst and D.W. Golde, Blood 84, 2912 (1994).
125. M.C. Dinauer, E.A. Pierce, R.W. Erickson, T.J. Muhlebach, H. Messner, S. Orkin, R.A. Seger and Cumutte, Proc. Natl. Acad. Sci. USA 88, 11231 (1991).
126. P. Finan, Y. Shimizu, I. Gout, J. Hsuan, O. Truong, C. Butcher, P. Bennett, M.D. Waterfield and S. Kellie, J. Biol. Chem. 269, 13752 (1994).
127. T.R. Burke, P. Russ and B. Lim, Synthesis , 1019 (1991).
128. C. Garbay-Jaureguiberry, I. McCort-Tranchepain, B. Barbe, D. Ficheux and B.P. Roques, Asymmetry 3, 637 (1992).
129. D.J. McNamara, E.M. Dobrusin, G. Zhu, S.J. Decker and A.R. Saltiel, Pept. Protein. Res. 42, 240 (1993).
130. T.J. Burke, M.S. Smyth, A. Otaka, M. Nomizu, P.P. Roller, G. Wolf, R. Case and S.E. Shoelson, Biochem. 33, 6490 (1994).
131. V. Mikol, G. Baumann, T.H. Keller, U. Manning and M.G.M. Zurini, J. Mol. Biol. 246, 344 (1995).
132. K. Yokote, S. Mori, K. Hansen, J. McGlade, T. Pawson, C.H. Heldin and W.L. Claesson, J. Biol. Chem. 269, 15337 (1994).
133. S.M. Domchek, K.R. Auger, S. Chatterjee, T.J. Burke and S.E. Shoelson, Biochem. 31, 9865 (1992).
134. M.R. Kuhne, Z. Zhao, J. Rowles, B.E. Lavan, S.H. Shen, E.H. Fischer and G.E. Lienhard, J. Biol. Chem. 269, 15833 (1994).
135. B. Ye, M. Yan, G. Wolf, S.E. Shoelson, P.R. Roller and T.E. Burke, J. Med. Chem. *in press*, (1995).
136. C. McGuigan, R.N. Pathirana, J. Balzarini and E. De Clercq, J. Med. Chem. 36, 1048 (1993).
137. S. Freeman, Chem. Commun. , 875 (1991).
138. D. Farquhar, S. Kham, M.C. Wilkerson and B.S. Andersson, Tetrahedron Lett. 36, 655 (1995).

Chapter 25. Programmed Cell Death Mediated by Members of the TNF Receptor Family

Bruce Beutler
Howard Hughes Medical Institute, Dallas, TX 75235-9050

Introduction - The immune system is among the most plastic of tissues. Inherent in any specific immune response is the rapid expansion of T-cell and B-cell clones to meet an infectious challenge. Nonspecific immune reactions may involve a five- to ten-fold increase in the number of circulating neutrophils, occurring within a few hours. In both cases, the cells must quickly be removed after the pathogen is cleared. And in both cases, removal is accomplished through programmed cell death, or apoptosis.

Apoptotic destruction of cells is characterized by cytoplasmic condensation and fragmentation of nuclear DNA. It is often punctuated with phagocytic removal of the dying cell by neighboring cells. The terminal events of apoptotic death are phenomenologically complex and therefore poorly understood; however, the signaling events that mediate apoptosis have come into sharper focus with the appreciation that, in many instances, apoptosis is initiated by signals that arise in the extracellular environment.

Several members of the TNF ligand and receptor families are now known to be of central importance in the apoptotic destruction of lymphocytes, and perhaps other cells as well (1,2). Indeed, three of the ligand family members were isolated as specific inducers of apoptosis (3-5). Of particular note, the two receptors for homotrimeric TNF and lymphotoxin (LT) -α (the 55 kDa and 75 kDa TNF receptors) and the Fas antigen, are known to be capable of initiating cytolysis, whereas the NGF receptor and CD40 molecule appear to prevent cytolysis in the presence of their respective ligands. The 55 kDa TNF receptor, the Fas antigen, and CD40 receptor each contain a specialized sequence within the cytoplasmic domain, known as a"death domain." In the first two of these proteins, the death domain has been shown to be functionally important in the initiation of apoptosis. A coherent picture of the biochemical events that follow receptor activation has now begun to emerge.

THE TNF AND FAS SYSTEMS

TNF was first isolated as a protein responsible for inducing hemorrhagic necrosis of transplantable tumors in animals (3,6). A product of endotoxin-activated macrophages, it was also noted to cause the death of many varieties of transformed cells in vitro (7), as did its close homolog, the lymphocyte protein LT-α (4,8). The cytolytic potential of both cytokines was enhanced by coincubation of their cellular targets with inhibitors of protein or RNA synthesis. The lysis produced by TNF and LT-α was associated with DNA fragmentation (9,10), as is typically observed in apoptotic death.

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

When cloned, TNF and LT-α were noted to be strikingly similar to one another. Both were homotrimeric proteins, and both engaged a common set of plasma membrane receptors. The 55 kDa TNF receptor was quickly associated with cytotoxic activity (11), and the 75 kDa TNF receptor was found to stimulate T-cell proliferation (12) (more recent data have suggested that the 75 kDa receptor also has cytotoxic activity, albeit relatively weak (13-15)). Virus-infected cells were shown to have enhanced susceptibility to lysis by TNF (16), and more intriguing still, poxviruses were found to encode TNF inhibitory proteins that act to prevent the binding of TNF to infected cells (17). Both poxviruses and adenoviruses were noted to encode inhibitors (the *crm*A protein of poxvirus, and the E1B l9K and E3 14.7K proteins of adenovirus) that block TNF action at a postreceptor level (18-21). Moreover, the 55 kDa TNF receptor was found to act through an unknown mechanism to limit the growth of a number of intracellular pathogens, including *Listeria (22)* and *Mycobacteria* (23).

The Fas antigen was discovered as the outgrowth of work that was thematically related to early studies with TNF and LT-α. Two independent groups of investigators screened monoclonal antibodies for an ability to trigger the apoptotic lysis of tumor cells (24,25). The Fas antigen (5,25), separately dubbed Apo-1 (26), was identified as an integral plasma membrane protein recognized by each of these antibodies. Given the similar actions of TNF and Fas antibody, it was speculated that the Fas antigen might comprise the receptor for TNF. This proved to be incorrect; however, the Fas antigen is a member of the TNF receptor family.

The *lpr* (lymphoproliferation) mutation of mice represents an insertional event (27) that abolishes expression of the Fas antigen (28). Animals carrying this mutation in homozygous form show striking accumulation of a polyclonal CD4-, CD8-, B220+ T-cell population within the peripheral lymphoid organs, and suffer from an autoimmune disorder often taken as a model of systemic lupus erythematosus (29). The *gld* (generalized lymphoproliferative disease) mutation of mice represents a defect of the Fas ligand gene (30), which encodes a trimeric, type II plasma membrane protein homologous to TNF (31). Such animals exhibit a lymphoproliferative syndrome identical to that observed in *lpr* homozygotes. Finally, the lpr^{cg} allele encodes a mutant form of Fas antigen in which signal transduction is abolished, yet expression of a Fas-ligand binding domain persists, thereby "neutralizing" much of the Fas ligand that is available (32). Probably for this reason, and animal of lpr^{cg}/+; *gld*/+ genotype is affected by lymphoproliferative disease. The mutation in the lpr^{cg} allele resides within the region encoding the death domain (28).

The Fas ligand/Fas antigen complex is, therefore, clearly required for apoptotic removal of a distinct lymphocyte subtype. The origin of these cells, and their normal function, remains a mystery. They are deleterious if permitted to accumulate.

The CD40 molecule is also considered to have a death domain homolog (5). Mutations of CD40 or the CD40 ligand, however, cause an immunodeficiency state in which there is a failure of immunoglobulin switching and B-cell proliferation, rather than a defect characterized by a failure of apoptosis (33). For this reason, as in the similar case of the NGF receptor, it has been considered that unoccupied receptors might

mediate apoptosis, whereas ligand-occupied receptors might not transduce a killing signal (34).

THE TNF RECEPTOR IS A PREFORMED DIMER ON THE CELL SURFACE

Early models of TNF receptor activation stressed the importance of "dimerization" or "aggregation" of the receptor at the cell surface. It now appears that the TNF receptor- and by implication, other members of the receptor family- are dimers to begin with, and upon ligand binding, undergo conformational changes that lead to signal initiation. The distinction may seem a subtle one, but the model of a preformed dimer is far more consistent with the notion that each receptor "unit" comprises an independent molecular "switch."

Chimeric TNF receptors, in which the native extracellular domain was replaced with the erythropoietin (Epo) receptor extracellular domain, signal constitutively, causing cytolysis of cells in which they are expressed, whether the 55 kDa or 75 kDa cytoplasmic domain was present in the construct (15). Similarly, it was shown that a truncated version of the 55 kDa TNF receptor, in which the cytoplasmic domain alone was represented, has cytolytic potential (35). Moreover, work with the yeast 2-hybrid system revealed that the cytoplasmic domains of the 55 kDa receptor interact with one another, forming a dimer (35,36).

The dimeric structure of the 55 kDa receptor extracellular domain, crystallized in the absence of ligand, was most directly and clearly established by Sprang and coworkers (37). The extracellular domain was found to exist as a parallel ("head-to-head") dimer, with substantial buried surface suggestive of a relatively strong interaction between subunits. In separate studies, Banner *et al.* established the structure of the 55 kDa receptor extracellular domain co-crystallized with the ligand LT-α (38). The overall shape of each extracellular domain subunit was similar, whether the receptor was crystallized in the presence or absence of ligand. However, the relative orientation of the subunits with respect to one another was drastically altered: ligand binding apparently disrupts the intersubunit contacts that exist in the resting receptor, and causes rotation of one subunit through 240° with respect to the 2-fold axis of symmetry that defines the dimer in its resting state. It is this conformational change that corresponds to "throwing the switch." It is not clear whether this event is readily reversible.

Many perturbations of the receptor are apparently capable of initiating a signal. Ligand, anti-receptor antibody (39), substitution of an Epo receptor extracellular domain for the native extracellular domain (15), or deletion of the extracellular domain (35) are each sufficient to initiate cytolysis. From this standpoint, it might be said that the principal function of the TNF receptor extracellular domain is to prevent signal transduction in the absence of ligand.

It may be assumed that the Fas receptor and the 55 kDa receptor function in a similar way; i.e., as simple mechanical switches. The CD40 molecule and the NGF receptor may be different, in that it appears that signal transduction may proceed in

the absence of ligand, and be terminated in its presence. It seems probable, however, that they also exist as preformed dimers that undergo conformational change in the presence of ligand.

THE CYTOPLASMIC SIDE

Recent studies have begun to reveal proteins that engage TNF receptor family members beneath the cell membrane. All have been identified by use of the yeast 2-hybrid system, which has proven to be an invaluable tool in the detection of protein:protein interaction. It is probable that these proteins are involved in signal transduction. Three general classes of interactive proteins have emerged:

Ring- and Zinc-Finger Proteins - Rothe *et al.* (40) first identified two proteins that engage the cytoplasmic domain of the 75 kDa TNF receptor, and noted that their sequences contained both ring-finger and the more classical C2H2 zinc-finger motifs. Termed "TRAFs" (TNF Receptor Associated Factors), these proteins had no obvious similarity to any proteins reported prior to that time. Shortly thereafter, however, CRAF-1 and CRAF-2 were identified as proteins that engage the cytoplasmic domain of the CD40 receptor (41). And most recently, the protein LAP-1 was identified as a factor that binds both to the LMP- 1 protein of Epstein-Barr virus (a plasma membrane protein known to promote the mitogenesis of infected B-cells) and to the cytoplasmic domains of the 75 kDa TNF receptor, the LT-β receptor, and the CD40 molecule (42). The suggestion that these proteins are essential and early components of the signal transduction cascade has yet to be confirmed. However, it is particularly compelling to note that a viral protein involved in mitogenesis has come to rely upon association with a cellular factor that may be required for the same effect, transduced by extracellular ligands.

FADD - A unique protein that engages the Fas antigen cytoplasmic domain is FADD, so named because like the Fas antigen and 55 kDa TNF receptor, it contains a death domain homolog within its C-terminal region (43). FADD forms heterodimers with the Fas antigen cytoplasmic domain through interactions involving the death domain. Point mutations corresponding to inactivating mutations in Fas abolish heterodimer formation. If over expressed as a full-length protein, FADD causes cell death. Over expression of the amino-terminal half of the molecule, which lacks the death domain, also causes cell death.

Protein Tyrosine Phosphatase (and Kinases, and Others) - An additional protein identified in physical association with the Fas antigen is a protein tyrosine phosphatase (FAP-1), the downstream target of which is unknown (44). It has also been reported that a serine kinase is closely associated with the 55 kDa TNF receptor, although this molecule has yet to be isolated (45). Finally, a molecule vaguely related to the 90 kDa heat shock protein has been found to bind to the 55 kDa TNF receptor cytoplasmic domain (46), although the significance of this interaction has not been determined.

CONDUCTION OF THE LETHAL SIGNAL INTO THE CYTOPLASM

Several proteins of *C. elegans* are now recognized as important regulators of cell death during development (47). The mammalian homolog of ced-3 is the interleukin-1β converting enzyme (ICE) (48). The mammalian homolog of ced-9 is Bcl-2 (49,50). By analogy, ICE and Bcl-2 might be expected to fulfill regulatory roles in mammalian cell apoptosis.

Bcl-2 was first identified as gene product over expressed as the result of a translocation event in certain B-cell lymphomas (51). It assures the immortality of cells which would otherwise succumb to programmed death. Heterodimerization between Bcl-2 and related proteins such as Bax and Bak leads to inactivation of Bcl-2, permitting apoptotic death. On the other hand, homodimerization of Bcl-2, or heterodimerization between Bcl-2 and the unrelated protein Bag-1, each lead to enhanced cell survival (52). Of importance to apoptosis induced by TNF and Fas ligand, it is clear that over expression of Bcl-2 protects cells against death (53-55). Still more remarkably, Bak, which inactivates Bcl-2, appears is specifically engaged by the adenovirus E1B 19K protein (56), which is known to block the cytolytic effect of TNF (57).

The connection between ICE and apoptosis is a more tenuous one. ICE is homologous to ced-3 and over expression of ICE induces apoptosis in cultured cells (58). Moreover, the *crm*A protein encoded by poxvirus directly interacts with ICE, and blocks apoptosis mediated by TNF and anti-Fas antibody (18). It has been noted that Bag-1, which enhances the protective activity of Bcl-2, contains a ubiquitin-like domain (52), and so, might act as a target for proteolytic attack.

However, ICE-deficient mice have no obvious defect of apoptosis (59). Most certainly, they do not develop lymphoproliferative disease, indicating that if ICE conducts the apoptotic signal initiated by the Fas antigen, it fulfills a function that is duplicated by other proteins. Conceivably, yet-to-be-identified homologs of ICE might bypass the lesion imposed by ICE gene knockout. Alternatively, the finding that ICE over expression causes apoptosis might reflect an effect that is unphysiologic, and the association of *crm*A with ICE might be fortuitous.

The involvement of other proteins in apoptosis has also been proposed. Notably, neutral sphingomyelinase is induced by TNF, and has been cited as the proximal source of ceramide, a metabolite which induces apoptosis when added directly to cultured cells.

SUMMARY

Cell death can, at times, be more vital to the organism than cell life. Witness the severity of disease that is caused by a discrete failure of apoptosis (in mice homozygous for the *lpr* or *gld* mutations), as compared with the severity of disease caused by total T-cell aplasia (in mice homozygous for the *nu* mutation). Arguably, lymphoproliferation is a more serious problem. There is reason to believe that many

autoimmune diseases might result from similar failures of apoptosis, and concomitant survival of an immune response beyond its useful lifetime. The TNF receptors and their homologs act as discrete molecular switches in this process, and in understanding the signal transduction apparatus that serves them, it is likely that much might be learned of perplexing diseases. Further, a more sophisticated approach to the induction of apoptosis might be fashioned with therapeutic intent.

REFERENCES

1. B. Beutler and C. Van Huffel, Ann. NY Acad. Sci., 730, 118 (1994).
2. B. Beutler and C. Van Huffel, Science, 264, 667 (1994).
3. D. Pennica, G.E. Nedwin, J.S. HayElick, P.H. Seeburg, R. Derynck, M.A. Palladino, W.J. Kohr, B.B. Aggarwal, and D.V. Goeddel, Nature, 312, 724 (1984).
4. P.W. Gray, B.B. Aggarwal, C.V. Benton, T.S. Bringman, W.J. Henzel, J.A. Jarrett, D.W. Leung, B. Moffat, P. Ng, L.P. Svedersky, M.A. Palladino, and G.E. Nedwin, Nature, 312, 721 (1984).
5. N. Itoh, S. Yonehara, A. Ishii, M. Yonehara, S. Mizushima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata, Cell, 66, 233 (1991).
6. E.A. Carswell, L.J. Old, R.L. Kassel, S. Green, N. Fiore, and B. Williamson, Proc. Natl. Acad. Sci., USA., 72, 3666 (1975).
7. L. Helson, S. Green, E. Carswell, and L.J. Old, Nature, 258, 731 (1975).
8. G.A. Granger and T.W. Williams, Nature, 218, 1253 (1968).
9. D. S. Schmid, J.P.Tite, and N.H. Ruddle, Proc. Natl. Acad. Sci., USA., 83, 1881 (1986).
10. D.S. Schmid, R. Hornung, K.M. McGrath, N. Paul, and N.H. Ruddle, Lymphokine. Res., 6, 195 (1987).
11. B.Thoma, M. Grell, K. Pfizenmaier, and P. Scheurich, J. Exp. Med., 172, 1019 (1990).
12. L.A. Tartaglia, D.V. Goeddel, C. Reynolds, I.S. Figari, R.F. Weber, B.M. Fendly, and M.A. Palladino, Jr., J.Immunol., 151, 4637 (1993).
13. J. Bigda, I. Beletsky, C. Brakebusch, Y. Varfolomeev, H. Engelmann, H. Holtmann, and D. Wallach, J. Exp. Med., 180, 445 (1994).
14. A.E. Medvedev, A. Sundan, and T. Espevik, Eur. J. Immunol., 24, 2842 (1994).
15. F. Bazzoni, E. Alejos, and B. Beutler, Proc. Natl. Acad. Sci., USA., in press, (1995).
16. W.C. Koff and A.V. Fann, Lymphokine Res., 5, 215 (1986).
17. S.T. Howard, Y.S. Chan, and G.L. Smith, Virology, 180, 633 (1991).
18. M. Tewari and V.M. Dixit, J. Biol. Chem., 270, 3255 (1995).
19. L.R. Gooding, L.W. Elmore, A.E. Tollefson, H.A. Brady, and W.S.M. Wold, Cell, 53, 341 (1988).
20. T. S . Ranheim, J. Shisler, T.M. Horton, L.J. Wold, L.R. Gooding, W.S.M. Wold, J. Virol., 67, 2159 (1993).
21. J.M. Tufariello, S. Cho, and M. S. Horwitz, Proc. Natl. Acad. Sci. USA., 91, 10987 (1994).
22. E.A. Havell, J. Immunol., 143, 2894 (1989).
23. V. Kindler, A. Sappino, G.E. Grau, P. Piguet, and P. Vassalli, Cell, 56, 731 (1989).
24. B.C. Trauth, C. Klas, A.M.J. Peters, S. Matzku, P. Moller, W. Falk, K. Debatin, and P.H. Krammer, Science, 245, 301 (1989).
25. S . Yonehara, A. Ishii, and M. Yonehara, J. Exp. Med., 169, 1747 (1989).
26. A. Oehm, I. Behrmann, W. Falk, M. Pawlita, G. Maier, C. Klas, M. Li-Weber, S. Richards, J. Dhein, B.C. Trauth, H. Ponstingl, and P.H. Krammer, J. Biol. Chem., 267, 10709 (1992).
27. M. Adachi, R. Watanabe-Fukunaga, and S. Nagata, Proc. Natl. Acad. Sci., USA., 90, 1756 (1993).
28. R. Watanabe-Fukunaga, C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata, Nature, 356, 314 (1992).

29. S. Izui, V.E. Kelley, K. Masuda, H. Yoshida, J.B. Roths, and E.D. Murphy, J. Immunol., 133, 227 (1984).
30. T. Takahashi, M. Tanaka, C.I. Brannan, N.A. Jenkins, N.G. Copeland, T. Suda, and S. Nagata, Cell, 76, 969 (1994).
31. T. Suda, T. Takahashi, P. Golstein, and S. Nagata, Cell, 75, 1169 (1993).
32. A. Matsuzawa, T. Moriyama, T. Kaneko, M. Tanaka, M. Kimura, H. Ikeda, and T. Katagiri, J. Exp. Med., 171, 519 (1990).
33. R.E. Callard, R.J. Armitage, W.C. Fanslow, and M.K. Spriggs, Immunol. Today, 14, 559 (1993).
34. S. Rabizadeh, J. Oh, L. Zhong, J. Yang, C.M. Bitler, L.L. Butcher, and D.E. Bredesen, Science, 261, 345 (1993).
35. M.P. Boldin, I.L. Mett, E.E. Varfolomeev, I. Chumakov, Y. Shemer-Avni, J.H. Camonis, and D. Wallach, J. Biol. Chem., 270, 387 (1995).
36. H.Y.Song,J.D.Dunbar, and D.B.Donner, J. Biol. Chem., 269, 22492 (1994).
37. J.H. Naismith, T.Q. Devine, B.J. Brandhuber, and S.R. Sprang, J. Biol. Chem., in press (1995).
38. D.W. Banner, A. D'Arcy, W. Janes, R. Gentz, H. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer, Cell, 73, 431 (1993).
39. H. Engelmann, H. Holtmann, C. Brakebusch, Y. S. Avni, I. Sarov, Y. Nophar, E. Hadas, O. Leitner, and D. Wallach, J. Biol. Chem., 265, 14497 (1990).
40. M. Rothe, S.C. Wong, W.J. Henzel, and D.V. Goeddel, Cell., 78, 681 (1994).
41. H.M. Hu, K. O'Rourke, M.S. Boguski, and V.M. Dixit, J. Biol. Chem., 269, 30069 (1994).
42. G. Mosialos, M. Birkenbach, R. Yalamanchili, T. VanArsdale, C. Ware, and E. Kieff, Cell, 80, 389 (1995).
43. A.M. Chinnaiyan, K. O'Rourke, M. Tewari, and V.M. Dixit, Cell, in press, (1995).
44. T. Sato, S. Irie,S.Kitada, and J.C. Reed, Science, 268, 411 (1995).
45. T.L. VanArsdale and C.F. Ware, J. Immunol., 153, 3043 (1994).
46. H.Y. Song, J.D. Dunbar, Y.X. Zhang, D. Guo, and D.B. Donner, J. Biol. Chem., 270, 3574 (1995).
47. R.E. Ellis, J.Y. Yuan, and H.R. Horvitz, Annual Review of Cell Biology, 7, 663 (1991).
48. J. Yuan, S. Shaham, S. Ledoux, H.M. Ellis, and H.R. Horvitz, Cell, 75, 641 (1993).
49. M.O. Hengartner, R.E. Ellis, and H.R. Horvitz, Nature, 356, 494 (1992).
50. D.L.Vaux, I.L.Weissman, and S.K. Kim, Science, 258, 1955 (1992).
51. S.Tsujimoto, and C.M.Croce, Proc. Natl. Acad. Sci., USA., 83, 5214 (1986).
52. S.Takayama, T. Sato, S. Krajewski, K. Kochel, S. Irie, J.A. Millan, J.C. Reed, Cell, 80, 279 (1995).
53. B. Vanhaesebroeck, J.C. Reed, D. De Valck, J. Grooten, T. Miyashita, S. Tanaka, R. Beyaert, F. Van Roy, and W. Fiers, Oncogene, 8, 1075 (1993).
54. H. Albrecht, J. Tschopp, and C.V. Jongeneel, FEBS.Lett., 351, 45 (1994).
55. T. Hennet, G. Bertoni, C. Richter, and E. Peterhans, Cancer Res., 53, 1456 (1993).
56. S.N. Farrow, J.H.M. White, I. Martinou, T. Raven, K.-T. Pun, C.J.Grinham, J.C. Martinou, and R. Brown, Nature, 374, 731 (1995).
57. L.R. Gooding, L. Aquino, P.J. Duerksen-Hughes, D. Day, T.M.Horton, S.Yei, and W.S.M. Wold, J.Virology, 65, 3083 (1991).
58. M. Miura, H. Zhu, R. Rotello, E.A. Hartwieg, and J. Yuan, Cell, 75, 653 (1993).
59. P. Li, H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, E. Towne, D.Tracey, S. Wardwell, F.-Y. Wei, W. Wong, R. Kamen, and T. Seshadri, Cell, 80, 401 (1995).

Chapter 26. Tyrosine Kinase Induced Mitogenesis Breaking the Link With Cancer

Mikhail L. Gishizky
SUGEN, Inc., 515 Galveston Dr., Redwood City, CA 94063

Protein tyrosine kinases (TK) play a critical role in mediating cellular responses to environmental signals. TKs are known to effect a broad range of cellular processes including proliferation, differentiation, migration, metabolism and cell death. Alteration in the activity of TKs can lead to profound biological consequences and are associated with a number of human diseases including cancer, diabetes and immune dysfunctions.

Protein tyrosine kinases can be segregated into two general classes, receptor (RTK) and non-receptor (non-RTK). It appears that activation of both classes of tyrosine kinases may require dimerization (1). In the case of RTKs, such as epidermal growth factor receptor (EGFR), ligand binding to the extracellular domain induces dimerization which allows the internal tyrosine kinase domains to trans-phosphorylate tyrosine residues. The phosphorylated tyrosines then become binding sites for proteins containing SH2 (src homology 2) domains (2) or the recently described PID domain (phosphotyrosine interaction domain) (3,4). The amino acid context surrounding the phosphotyrosine determines which specific phosphotyrosine binding protein will interact with the receptor. Once bound to the RTK, the proteins initiate intracellular signaling cascades by either binding to and recruiting other intracellular signaling molecules or through their own intrinsic catalytic activity (5). This chapter will summarize the current understanding of the intracellular signaling pathways activated by protein tyrosine kinases. Particular emphasis will be given to the mitogenic pathway and its role in cancer.

The Link Between Tyrosine Kinases And Ras Activation - Ras, a small GTP-binding protein, is an important element in the signal transduction pathway which directs cells to proliferate and differentiate. Activation of RTKs or non-RTKs induces Ras to move from an inactive GDP-bound state to an active GTP-bound state. Studies using invertebrate and mammalian cell systems have elucidated the components of one pathway which links activated tyrosine kinases to Ras activation (Fig 1).

In the past year several articles have described in detail the intracellular signaling components which connect RTKs and Ras activation (2,6). In the case of EGFR, ligand induced dimerization results in trans-phosphorylation of specific phosphotyrosines that provide binding sites for the adaptor protein called Grb2 (Growth factor receptor binding protein 2). Adaptor proteins are a family of molecules that are composed of domains that allow them to interact with other proteins but themselves do not contain enzymatic activity (7). In effect they function as a bridge that brings together an enzyme and its substrate.

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

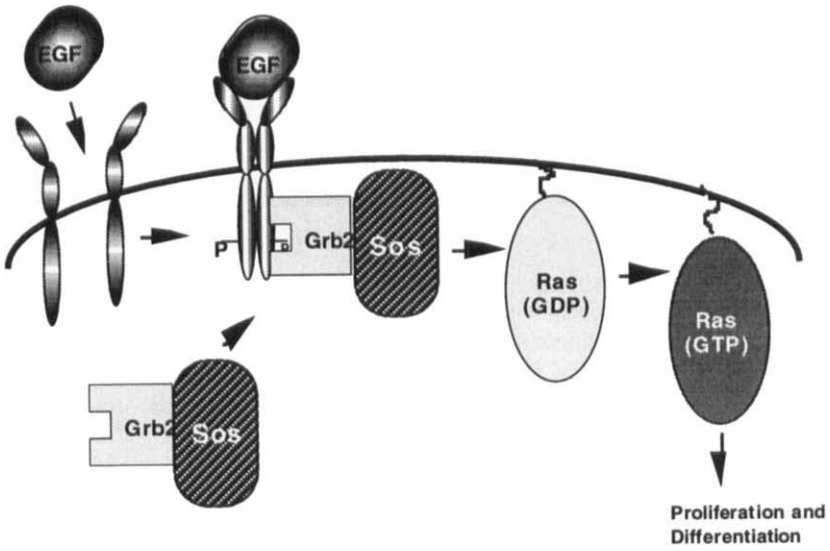


Fig. 1 Schematic representation of the intracellular signaling pathway involved in EGFR induced Ras activation.

In the unstimulated cell, the Grb2 protein appears to exist in a complex with the guanine nucleotide exchange factor Sos (son of sevenless). Phosphorylation of the EGFR results in the recruitment of the Grb2/Sos complex to the membrane proximal, cytoplasmic tail of the EGFR. The translocation of the associated Sos protein from the cytoplasm to the membrane facilitates interaction of Sos with the membrane associated Ras protein. The association of Sos with Ras results in the activation of Ras by mediating the conversion of GDP-bound Ras (inactive) to GTP-bound Ras (active). Recent studies have demonstrated that the mere presence of Sos in the membrane is sufficient to activate Ras (8).

Activation of Ras can also be induced by non-RTKs. Although the exact mechanisms which activate the non-RTKs are still unknown, the signaling components linking the activated non-RTK's to Ras appear to be the same as those described for RTK's (2,8). For example the non-RTK src contains a myristilation sequence at its amino terminus which promotes its association with the plasma membrane. Once activated, the tyrosine kinase activity of src phosphorylates the adaptor protein Shc. The phosphorylation of Shc on a particular tyrosine provides a binding site for the Grb2/Sos complex. The result once again, is the localization of Sos to the membrane where Ras is located. Together, these studies point to a linear pathway of signaling events from RTKs and non-RTKs to Ras activation, which is evolutionarily conserved between such phylogenetically diverse species as worms, fruit flies and man.

Ras Activation Leads To Proliferation And Differentiation - A major focus in the field of signal transduction has been to identify mechanisms responsible for directing a cell to proliferate rather than differentiate. An attractive

hypothesis is that distinct signaling pathways may exist for proliferation versus differentiation. However, recent studies suggest that this may not be the case (9).

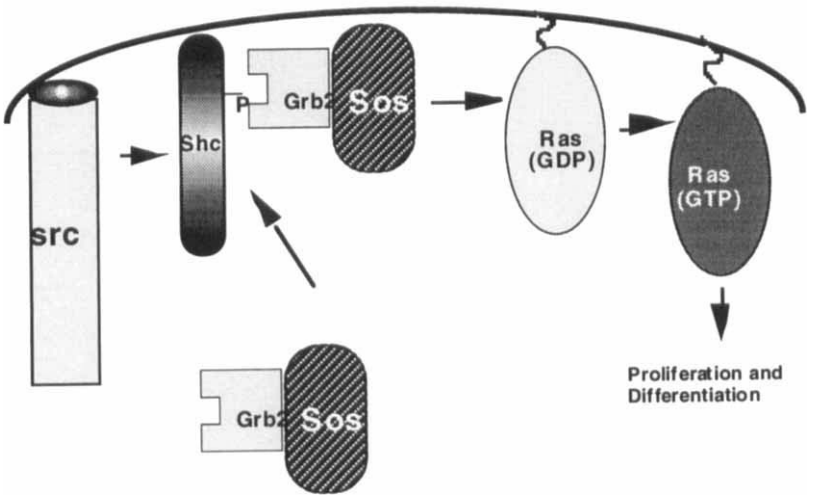


Fig. 2 Schematic representation of the intracellular signaling pathway linking non-receptor protein tyrosine kinase src to Ras activation.

There has been a long standing observation that factors which induce cells to proliferate and those that induce cells to differentiate both activate Ras. Injection of oncogenic (constitutively activated) Ras protein into quiescent fibroblasts stimulates DNA synthesis in the absence of any other mitogenic signals (10,11). Oncogenic Ras can also mimic the effect of nerve growth factor (NGF) in stimulating the outgrowth of neurites in PC12 cells (12-14). These apparently paradoxical observations fueled the intensive effort to elucidate the signaling pathways activated by Ras.

Ras activation has been coupled to the activation of two distinct effector pathways composed of mitogen activated protein kinases (MAPK), generically referred to as "MAPK" pathways (15,16). These signaling pathways are comprised of a three component protein kinase cascade (Fig. 3) consisting of a serine/threonine protein kinase (i.e. Raf), which phosphorylates and activates a dual specificity protein kinase (i.e. Mek is capable of phosphorylating serine/threonine and tyrosine), which in turn phosphorylates and activates another serine/threonine protein kinase, extracellular signal-regulated kinase (Erk). Most attention has focused on the Ras/Raf/Mek/Erk pathway responsible for activation of the Erk members in the MAPK family. The Ras/Raf/Mek/Erk pathway is activated by RTKs and non-RTKs and appears to be important in both cellular differentiation and proliferation. In the past year the signaling elements involved in the second MAPK pathway (Ras/Mekk/Sek/Jnk[Sapk]) have been elucidated (17-20). The Jnk/Sapk pathway becomes activated in response to a range of cellular stress stimuli including UV irradiation, heat shock and tumor necrosis factor. This pathway is also activated by mitogens signaling through RTK's such as EGFR. Based on work in lower eukaryotic organisms, it is clear that still other as yet undefined MAPK pathways must also be present in mammalian cells. The

identification of these multiple pathways underscores the complexity of the intracellular signaling circuitry and brings to light the critical question of how a cell may achieve specificity down parallel signaling cascades.

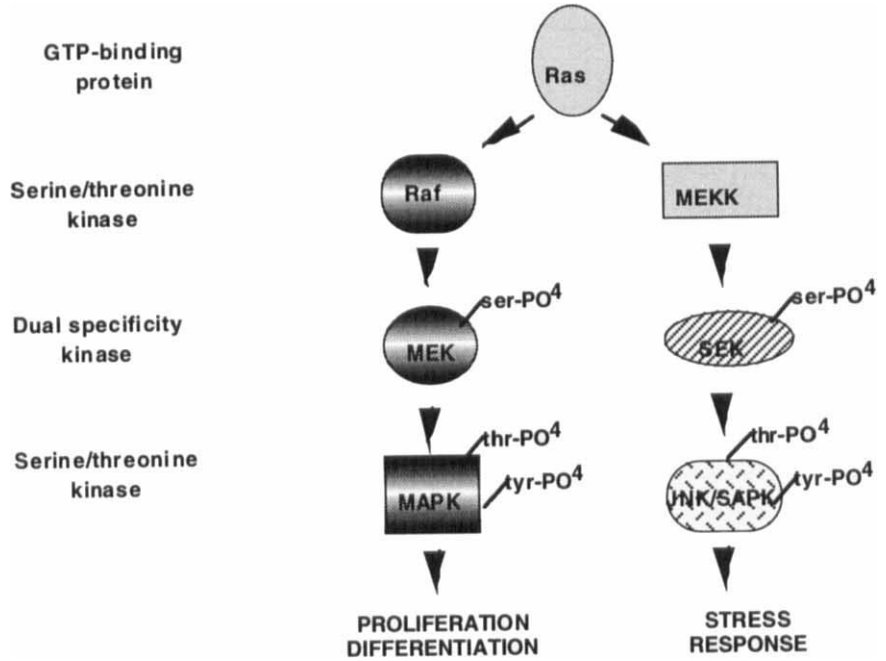


Fig. 3 Schematic representation of parallel MAPK signaling pathways.

PC12 cells have been extensively used as a model system for investigating how the activation of some receptors leads to differentiation while the activation of others induces proliferation. Treatment of PC12 cells with NGF results in cessation of growth and induction of neurite outgrowth (21). In contrast, treatment with epidermal growth factor induces a proliferative response (22). A qualitative analysis of the intracellular signaling pathways activated by NGF versus EGF revealed that both ligands appeared to activate the same pathways. However, closer analysis revealed that these ligands induced quantitative differences in the activation of specific cellular signaling components. NGF stimulation results in a persistent elevation of Ras-GTP within the cell, which is commensurate with a sustained activation of ERK (23). In contrast, EGF stimulation produces a transient elevation of Ras-GTP and commensurate short lived activation of ERK (24-26). These correlative observations have led to the idea that sustained activation of the Ras/Raf/Mek/Erk pathway may lead to differentiation (27). Additional support for this idea has come from studies using activating or inhibitory mutant forms of the Mek protein (27). Expression of an activated form of Mek in PC12 cells leads to the phosphorylation of Erk and induces neurite outgrowth in the absence of differentiation factors. Whereas, expression of the interfering mutant of Mek inhibits activation of Erk in the presence of differentiation factors and blocks differentiation (27). Together, these studies suggest that the duration of Erk activation may determine whether PC12 cells proliferate or differentiate (9).

From the preceding discussion it is clear that activation of multiple intracellular protein kinase cascades may be necessary to elicit a full biological response. The multiple parallel pathways may serve to amplify the signal being sent by a specific stimulus (i.e. TK). The relative contribution of each of these pathways to the end phenotype (either proliferation or differentiation) may vary depending on the specific cell type being studied. This is a particularly important consideration when evaluating intracellular signaling components as potential therapeutic targets.

The Adaptor Protein Grb2. A Target For Cancer Therapeutics - Activated protein tyrosine kinases have been implicated in a broad range of human cancers. One of the best characterized examples of a tyrosine kinase playing a causative role in human malignancy is that of the chimeric bcr/abl oncogene in human chronic myelogenous leukemia (CML). BCR/ABL is a cytoplasmic tyrosine kinase that is formed by the translocation of sequences from cabl tyrosine kinase gene on chromosome 9 into the bcr gene on chromosome 22. This abnormal chromosomal translocation, termed the Philadelphia chromosome (Ph1), is found in 95% of the patients that present with CML. The BCR/ABL protein exhibits elevated levels of tyrosine kinase activity and is able to induce mitogenesis and transformation of fibroblasts and hematopoietic cell lines. BCR/ABL, when expressed in murine hematopoietic cells *in vitro*, can induce growth factor independent growth and when expressed *in vivo*, can induce a murine leukemic disorder that closely resembles human CML.

The ability of BCR/ABL to induce cellular transformation correlates with its ability to activate Ras. The intracellular components of at least one pathway which links BCR/ABL to Ras have been identified (28). Not surprisingly, this pathway consists of the same elements described for other tyrosine kinases, specifically the proteins Grb2 and Sos. Two groups have shown that the Grb2/Sos complex can directly bind to BCR/ABL and that formation of the BCR/ABL-Grb2/Sos complex results in the activation of Ras (28, 29). Although additional pathways leading from BCR/ABL to Ras may exist, these observations suggested that by interfering with the formation of the BCR/ABL-Grb2/Sos complex, one could break the link between tyrosine kinase and Ras activation (28). To test this hypothesis mutant Grb2 proteins have been made and evaluated for their ability to block and reverse BCR/ABL induced transformation (30).

The Grb2 protein consists of an SH2 (src homology 2) domain bracketed by SH3 (src homology 3) domains, one each at the amino and carboxy terminus. The structure of the Grb2 protein indicates that each of these SH2 and SH3 domains has the potential to function as a separate binding entity (5). The Grb2 SH2 domain preferentially binds to a phosphorylated tyrosine residue in the context of tyrosine-valine-asparagine-valine (YVNV) present on BCR/ABL. The SH3 domains of Grb2 bind to proline rich sequences present on the Sos protein. Grb2 mutant proteins containing deletions of either the amino or carboxy terminus SH3 domains were expressed in BCR/ABL transformed and normal fibroblasts. Expression of the mutant Grb2 proteins in non-transformed fibroblasts had no apparent effect on cell growth (30). In contrast, expression of Grb2 mutant proteins in BCR/ABL transformed cells reversed the transformed phenotype (30). Furthermore, expression of the

Grb2 proteins in K562 cells, a BCR/ABL transformed human hematopoietic cell line established from a CML patient, blocked the growth and allowed the differentiation of K562 cells in culture (30). The dramatic reversal of BCR/ABL induced transformation and lack of cellular toxicity associated with expression of the mutant Grb2 proteins suggests that the mutants do not completely block intracellular signaling, but rather, may function by decreasing the amplitude of the mitogenic stimulus induced by BCR/ABL. These studies demonstrate that inhibition of tyrosine kinase induced mitogenesis can be accomplished by blocking specific signal transduction pathways downstream of tyrosine kinase enzymatic activity.

These studies suggest that blocking protein-protein interactions between components of intracellular signaling pathways may selectively inhibit mitogenesis. Development of drugs which specifically intercalate into protein-protein interfaces is an area of very active research. The resolution of the structure for a variety of protein binding domains including SH2 and SH3 domains has provided some insight as to how these domains recognize their specific targets (5). Based on crystal and NMR structure analysis the phosphotyrosine binding SH2 domain appears to form a very small interface at the center of which is a pocket into which the phosphorylated tyrosine residue fits (31-35). The specificity of interaction between a particular SH2 domain and a phosphorylated tyrosine is determined by the amino acids directly adjacent to the tyrosine residue. The small protein interface and defined pocket structure is consistent with the relatively short amino acid sequence which dictates specificity that has been demonstrated for the SH2 domain binding interactions *in vitro* and *in vivo*. In contrast, the resolved structure of SH3 domains reveal a relatively large folded beta-sheet configuration. The protein interface of the SH3 domain resembles a groove which can simultaneously interact with multiple amino acid residues. The larger interface of the SH3 domain may explain the relatively promiscuous nature of the SH3 interaction observed *in vitro*. Although protein binding domains offer attractive targets for drug discovery, it remains to be seen whether small molecules can be designed which exhibit the necessary affinity and selectivity required for the treatment of human disease.

Summary - Over the past five years tremendous strides have been made towards identification of the intracellular components involved in signal transduction. The emerging theme from these studies is that cellular responses are regulated by the activity of multiple parallel signaling pathways. Furthermore, qualitative and quantitative differences in activities of specific pathways may be critical in defining the cellular response initiated by a tyrosine kinase. In the next five years, the challenge will be to understand the mechanisms responsible for integrating and prioritizing the multiple signals a cell receives from its environment. Although the age of "signal transduction therapy" is upon us, we have much to learn regarding the basics of the signaling pathways involved.

References

1. J. Schlessinger and A. Ullrich, Neuron 9, 383 (1992).
2. T. Pawson and J. Schlessinger, Current Biology 3, 434 (1993).
3. P. Bork and B. Margolis, Cell 80, 693 (1995).

4. W.M. Kavanaugh and L.T. Williams, Science 266, 1862 (1994).
5. T. Pawson, Nature 373, 573 (1995).
6. C. Wilson, Trends in Cell Biol., 4, 409 (1994).
7. T. Pawson and G.D. Gish, Cell 71, 359 (1992).
8. J. Schlessinger, Current Opin. Genet. Dev., 4, 25 (1994).
9. C.J. Marshall, Cell 80, 179 (1995).
10. D. Bar-Sagi and J.R. Ferasmico, Cell 42, 841 (1985).
11. J.D.H. Morris, B. Price, A.C. Lloyd, A.J. Self, C.J. Marshall, A. Hall, Oncogene 4, 27 (1989).
12. D. Bar-Sagi and J.R. Ferasmico, Cell 42, 841 (1985).
13. M. Noda, M. Ko, A. Ogura, D.G. Li, T. Amano, T. Tkano, Y. Ikawa, Nature 318, 73 (1985).
14. K.W. Wood, H. Qi, R.C. Armstrong, T.M. Roberts, S. Halegoua, Proc. Natl. Acad. Sci. USA, 90, 5016 (1993).
15. R.J. Davis, Trends in Biol. Sci., 19, 470 (1994).
16. E. Cano and L.C. Mahadevan, Trends in Biol. Sci., 20, 117 (1995).
17. M. Yan, T. Dai, J.S. Deak, J.M. Kyraikis, L.I. Zon, J.R. Woodgett, D.J. Templeton, Nature 372, 798 (1994).
18. I. Sanchez, R.T. Hughes, B.J. Mayer, K. Yee, J.R. Woodgett, J. Avruch, J.M. Kyraikis, L.I. Zon, Nature 372, 794 (1994).
19. T. Deng and M. Karin, Nature 371, 171 (1994).
20. A. Minden, A.Lin, M. McMahon, C. Lange-Carter, B. Derijard, R.J. Davis, G.L. Johnson, M. Karin, Science 266, 1719 (1994).
21. L.A. Greene and A.S. Tischler, Proc. Natl. Acad. Sci. USA 72, 2424 (1976).
22. K. Huff, D. End, G. Guroff, J. Cell Biol. 88, 189 (1981).
23. K. Muroya, S. Hattori, S. Nakamura, Oncogene 7, 277 (1992).
24. L.E. Heasley and G.L. Johnson, Mol. Cell. Biol. 3, 545 (1992).
25. S. Traverse, N. Gomez, H. Paterson, C. Marshall, P. Cohen, Biochem. J. 288, 351 (1992).
26. T.T. Nguyen, J.C. Scimeca, C. Filloux, P. Peraldi, J.L. Carpenter, E. van Obberghen, J. Biol. Chem. 268, 9803 (1993).
27. S. Cowely, H. Paterson, P. Kemp, C.J. Marshall, Cell 77, 841 (1994).
28. A.M. Pendergast, L.A. Quillam, L.D. Cripe, K. Rabun, C.J. Der, J. Schlessinger, M.L. Gishizky, Cell 75,175 (1993).
29. L. Puil, J. Liu, G. Gish, G. Mbamalu, D. Bowtell, P.G. Pelicci, R. Arlinghous, T. Pawson, EMBO J. 13, 764 (1994).
30. M.L. Gishizky, D. Cortez, A.M. Pendergast, Proc. Natl. Acad. Sci. USA, in press (1995).
31. G. Waksman, Cell Mol. Biol. 40, 611 (1994).
32. G.W. Booker, A.L. Breeze, A.K. Downing, G. Panayotou, I. Gout, M.D. Waterfield, I.D. Campbell, Nature 20, 684 (1992).
33. G. Waksman, S.E. Shoelsom, N. Pant, D. Cowburn, J. Kuriyan, Cell 72, 779 (1993).
34. M. Hensmann, G.W. Booker, G. Panayotou, J. Boyd, J. Linacre, M. Waterfield, I.D. Campbell, Protein Sci. 3, 1020 (1994).
35. S. Maignan, J.P. Guilloteau, N. Fromage, B. Arnoux, J. Becquart, A. Ducruix, Science 268, 291 (1995).

SECTION VI. TOPICS IN DRUG DESIGN AND DISCOVERY

Michael C. Venuti
Arris Pharmaceutical Corporation
South San Francisco, CA 94080

Chapter 27. Catalytic Antibodies

Thomas S. Scanlan
Department of Pharmaceutical Chemistry, University of California,
San Francisco, CA 94143-0446

Introduction - Antibodies are a family of binding proteins that mediate the humoral immune response in vertebrates (1,2). Their natural evolutionary function is to bind and neutralize foreign substances called antigens. Anitbodies are large 150 kD homodimeric proteins consisting of two identical 50 kD polypeptides (heavy chains) and two identical 25 kD polypeptides (light chains); two identical antigen binding sites are located at the N-terminal regions of the homodimer. Most of the antibody residues that make binding contacts with the antigen reside in six loop structures called complementary determining regions (CDR's). Three CDR's are from the light chain and the other three CDR's are from the heavy chain. The amino acid sequences of the CDR's is highly variable between antibody molecules, and it has been estimated that some 10^{12} different antibody molecules, and hence the potential for 10^{12} different antigen-binding specificities, exists within a single organismal immune response. Because of this structural diversity, antibodies are able to bind a wide variety of antigen molecules, ranging from small organic compounds to larger proteins, viruses, and cell surface antigens.

Enzymes are proteins that have evolved to catalyze biochemical reactions (3). They do so by stabilizing the transition state of the reaction, thus providing a lower energy pathway for conversion of substrate to product. Accordingly, it has been proposed that the active site structure of an enzyme has evolved to be more complementary to the transition state structure rather than the ground state structures of substrates or products (4,5). Given the adaptable nature of the antibody molecule, it is perhaps not surprising that antibodies that bind transition state (TS) analogs can be produced from the vertebrate immune response. It has been shown that these antibodies, called catalytic antibodies, behave similarly to enzymes in that they show saturation kinetics, substrate selectivity, and accelerate the rate of reaction over the uncatalyzed background reaction. As a technology, catalytic antibodies offer a potential source of laboratory-designed enzymes for possible applications as research tools or therapeutics.

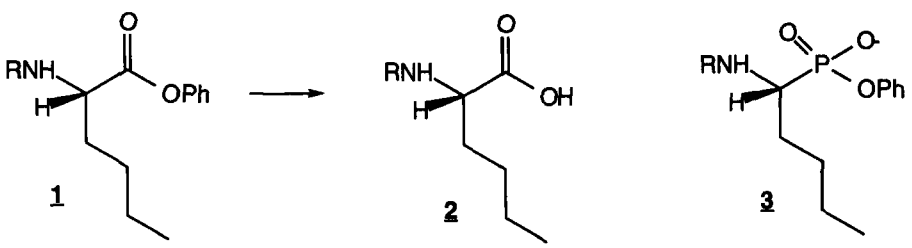
The experimental field of catalytic antibodies began in 1986 when the laboratories of Lerner and Schultz independently showed that antibodies specific for phosphate and phosphonate compounds (haptens) were catalysts for the hydrolysis of the corresponding carbonate substrates (6,7). In essence, these experiments verified a prediction made by Jencks some seventeen years earlier that antibodies with combining sites that were complementary to stable transition state analogs would be catalytically active in the same way as natural enzymes (8). The major advance that made the catalytic antibody proposal experimentally testable was the advent of monoclonal antibody technology in 1975 (9). This technique made it possible to obtain gram-quantities of homogeneous antibody samples which is crucial for the kinetic characterization of antibody catalysis.

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

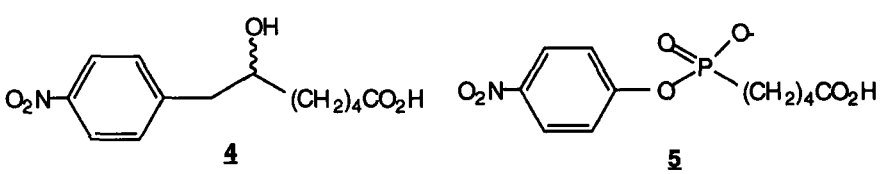
Since the intial reports from Lerner and Schultz, more than 100 papers have been published on the generation and characterization of new catalytic antibodies. The major research emphasis in the field over the past nine years has been to define the chemical scope of antibody catalysis and determine which classes of chemical reactions are amenable to antibody catalysis through transition state analog design (10). It is now clear that the scope of antibody catalysis is quite broad, and it is now reasonable to assume that any reaction for which a bona fide transition state analog can be synthesized will produce an antibody catalyst with some catalytic activity. A comprehensive list of the chemical reactions for which catalytic antibodies have been generated was published in 1991 (10). Several review articles covering different aspects of the field have been published (10-30). This review will discuss some of the TS analog designs that have led to esterolytic and amidolytic antibodies, and I will review new structure/mechanism studies and new methods for obtaining catalytic antibodies.

ESTERASE AND AMIDASE ANTIBODIES

Esterolysis - Ester hydrolysis has proven to be among the most general reaction types for antibody catalysis, and the most catalytically efficient esterase antibodies have come from phosphonate transition state analogs. For example, phosphonate **3** has been used to elicit an antibody that catalyzes the hydrolysis of norleucine phenyl ester analogs (**1**→**2**) with a k_{cat} of 223 min^{-1} and k_{cat}/K_M of 5 x 10^5 $M^{-1}min^{-1}$ (31). The methionine phenyl ester derivatives are also good substrates for this catalytic antibody affording similar k_{cat} values and K_M values that are approximately five times higher. In addition, this catalytic antibody has interesting structural and mechanistic properties that will be discussed in the next section.

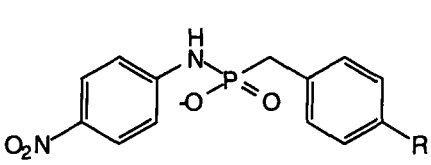


A second TS analog design that has had some success for eliciting esterolytic antibodies is the secondary alcohol structure. This design retains the tetrahedral geometry of the hydrolysis TS but does not include negative charge. In one case, a system was devised to compare directly the catalytic activity of antibodies raised to a secondary alcohol hapten with that of antibodies derived from a phosphonate hapten (32). Both hapten designs led to esterolytic antibodies that act on the same substrate, p-nitrophenyl acetate (PNA). The antibody raised to **4** catalyzes the hydrolysis of PNA with a k_{cat} of 0.72 min^{-1}, whereas the **5**-specific antibody has a k_{cat} for PNA hydrolysis of 7.4 min^{-1}. Thus, at least in this example, the phosphonate TS analog generates a catalytic antibody that is a ten-fold more active catalyst than the analogous catalyst obtained from the secondary alcohol TS analog.

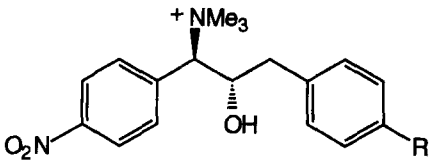


In another comparative study, the catalytic activity of an esterase antibodiy raised to phosphonmidate **6** proved to be about 10-fold higher than the esterase activity of an

antibody specific for the analogous TS-analog 7 (33,34). The hydroxy-ammonium TS-analog 7 was designed to mimic the tetrahedral geometry of the hydrolytic reaction center and to elicit an anionic active site residue to provide general acid/base catalysis. Monoclonal antibodies were elicited separately to both haptens and it was found that the a 6-specific antibody had approximately a 10-fold higher k_{cat}, again demonstrating a general superiority of the phosphonate TS analog over other hydrolytic TS analogs.

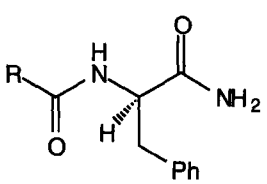


6

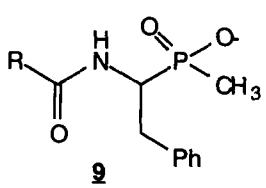


7

Amidolysis - Catalytic antibodies that can cleave peptide bonds in a site-specific manner would be very useful as research tools and potential therapeutics. For example, access to a family of site-specific proteases could facilitate structure-function studies on newly identified proteins, and proteolytic antibodies specific for viral coat proteins might be useful as antiviral therapeutics. However, amide hydrolysis has proven to be a much more difficult reaction for achieving antibody catalysis than ester hydrolysis. Despite the mechanistic similarity between amide and ester hydrolysis, amides are intrinsically more stable and the barriers to both formation and breakdown of the tetrahedral intermediate are much larger for amides than esters. Two examples of catalytic antibodies with modest amidase activity were recently reviewed (10); one antibody catalyzes the hydrolysis of a nitrophenyl anilide substrate, and was elicited with a phosphonamidate TS analog (35), and the other catalyzes peptide hydrolysis and was raised from a phosphonamidate-cobalt(III) TS analog complex (36). More recently, an antibody that catalyzes the hydrolysis of the unactivated phenylalanine amide substrate 8 has been reported (37). The antibody was elicited to the racemic phosphinate TS-analog 9 and is selective for the R-enantiomer of substrate 8. However, the catalytic activity of this antibody is modest with a k_{cat} of 1×10^{-5} min^{-1} and a rate acceleration (k_{cat}/k_{uncat}) of 132, again demonstrating the intrinsic difficulty of developing amide hydrolysis catalysts using TS analogs that have proven more successful in creating ester hydrolysis catalysts.



8



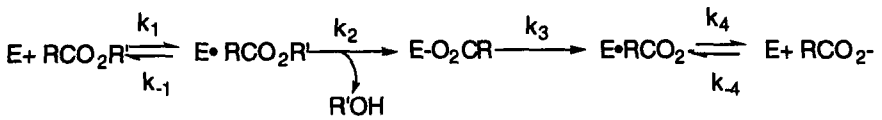
9

STRUCTURAL AND MECHANISTIC STUDIES

Recently a greater research emphasis has been placed on understanding antibody catalysis from a structural and mechanistic standpoint. The primary goal of these efforts is to use structural and mechanistic data to develop rules that will enable future TS analog designs to elicit more active catalytic antibodies, and to improve the activity of first-generation catalytic antibodies through site-directed mutagenesis (38-40). The esterase and amidase catalytic antibody, 43C9, is perhaps the most mechanistically studied catalytic antibody and a summary of the mechanistic properties of 43C9 has been recently published (26). A number of different mechanistic experiments suggest that 43C9 catalyzes ester and amide hydrolysis via a

sophisticated mechanism involving nucleophilic catalysis. To date, a three-dimensional structure has not been reported for 43C9. However, a structure based on computer modelling has been proposed (41). More recently, three crystal structures of catalytic antibodies have been solved, and these systems will be discussed in the following sections.

The 17E8 Phenylesterase System - The esterolytic antibody 17E8 was generated with the norleucine phosphonate TS analog **3** and catalyzes the hydrolysis of norleucine and methionine phenyl ester analogs (31). The catalysis of 17E8 is efficient (k_{cat}=223 min^{-1} for formylnorleucine phenylester at pH 9.5) and enantioselective for L-amino acid ester analogs. The 17E8-catalyzed esterolysis reaction is characterized by a bell-shaped pH-rate profile; maximum esterase activity occurs at pH 9.5, and apparent pK_a's of 9 and 10 are observed for the acidic and basic limbs of the profile respectively.



Results from steady-state hydroxylamine partitioning experiments are consistent with a multistep hydrolytic mechanism involving a covalent acyl intermediate analogous to the intermediate formed in serine protease-catalyzed esterolysis and amidolysis. The partitioning data suggest that either the acylation step (k_2) or the product release step (k_4) limits the rate of catalytic turnover.

The crystal structure of the 17E8 Fab fragment complexed with TS analog **3** has been solved to 2.5 Å resolution (42). The structure (Figure 1) shows that the L-enantiomer of **3** is bound exclusively in the active site even though a racemic mixture **3** was used in the crystallization experiment. This TS-analog binding enantioselectivity correlates with the observed L-enantioselectivity of 17E8-catalyzed esterolysis.

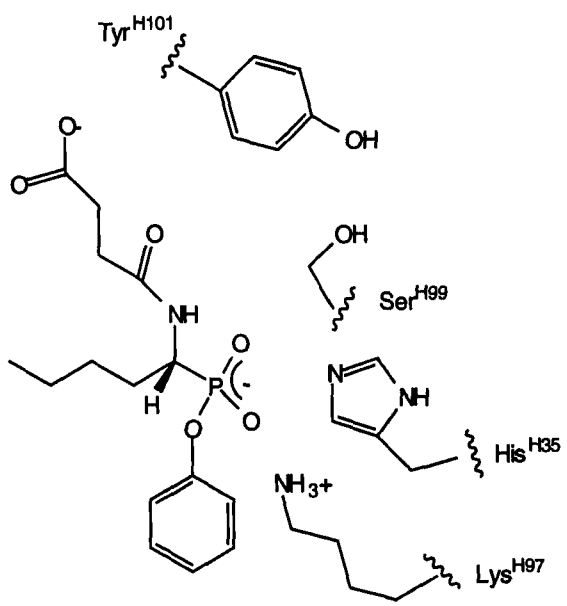


Figure 1: 17E8-**3** Active Site

The 17E8 active site contains two well-defined binding pockets for recognition of the phenyl ring and n-butyl side chain of **3**; the succinamide tether, which was used to attach **3** to an immunogenic carrier protein, extends out of the active site toward the solvent exposed region of the protein.

Candidate residues which directly participate in the 17E8-catalyzed esterolysis reaction are contained in the antibody heavy chain, and are grouped near the phosphonate moeity on one side of the active site. Lys^{H97} forms a salt-bridge interaction with the pro-S diastereotopic oxygen atom of **3**, suggesting that this electrostatic interaction is used to stabilize oxyanion development in the esterolysis transition state. This assignment is also consistent with the observed kinetic pK_a of 10 in the basic limb of the bell-shaped pH-rate profile. A pK_a of 10 is reasonable for a Lys ε-amino group, and if the protonated form of Lys^{H97} is responsible for oxyanion stabilization in the transition state, then a decrease in k_{cat} at pH > 9.5 would be expected.

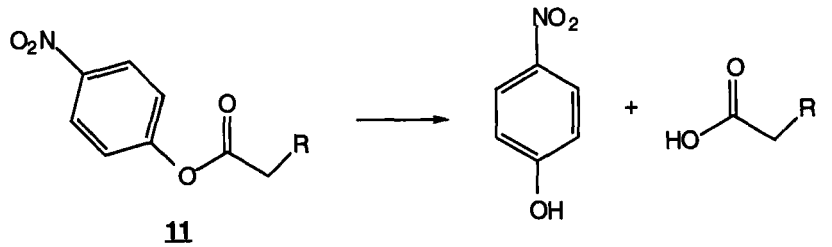
The simplest mechanistic explanation for the acidic limb of the pH-rate profile is that an active site residue with a pK_a of 9 accelerates catalysis in its deprotonated form, suggesting that this residue may function as a nucleophile or general base. A pK_a of 9 is reasonable for a phenolic proton of a Tyr residue; however, the active site structure of 17E8 shows that no Tyr residue is close enough to the bound TS-analog to function as either a nucleophile or a base. The closest Tyr residue is Tyr^{H101}, which appears to be involved in an H-bonding interaction with Ser^{H99}. Ser^{H99} is positioned between Tyr^{H101} and the P-atom of **3**, and the H-bonding interaction orients the Oγ of Ser^{H99} away from the P-atom of **3**.

This structural arrangement suggests that the ionization of Tyr^{H101} is the accelerating event that is seen in the acidic limb of the pH-rate profile, and the ionization disrupts the H-bond between Tyr^{H101} and Ser^{H99}, releasing the Oγ of Ser^{H99} to rotate and act as the nucleophile. Modelling a 180° rotation about Cα-Cβ of Ser^{H99} places Oγ about 3.0 Å away from the P-atom of **3** and within H-bonding distance to His^{H35}, setting up an active site arrangement that resembles two-thirds of the catalytic triad seen in serine protease enzymes (43). The modelled active site 17E8 structure can be overlayed on the active site of the trypsin-BPTI complex and the distances and geometries of the Ser, His, and inhibitors are remarkably similiar.

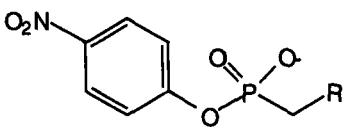
A second esterolytic antibody, 29G11, was obtained from the same anti-**3** monoclonal antibody panel as 17E8 (44). This antibody is also a fairly good catalyst for the hydrolysis of **2**, with a k_{cat} of 60 min^{-1}. The variable region polypeptide sequence of 29G11 suggests a strong structural similarity to 17E8; both antibodies possess identical light chains and the 29G11 heavy chain differs from that of 17E8 by nine point mutations. There is only one active site mutation where the nucleophilic residue, Ser^{H99}, in 17E8 is a non-nucleophilic Gly residue in 29G11. Because of this mutation, 29G11 shows kinetic behavior that is consistent with the 17E8 mechanistic model; The acidic limb of the 29G11 pH-rate profile is shifted toward neutral pH with an apparent pK_a of 8.1, and hydroxylamine partitioning experiments suggest that 29G11-catalyzed esterolysis does not involve nucleophilic catalysis.

The CNJ206 nitrophenylesterase - The esterolytic antibody CNJ206 was elicited from the nitrophenyl phosphonate TS analog **12** and catalyzes the hydrolysis of nitrophenyl ester **11** with a k_{cat} of 0.41 min^{-1} and rate acceleration of 1600 (45). The crystal structure (3.0 Å) of the CNJ206 Fab fragment in the unliganded form has recently been reported (46). In the absence of direct structural data, a model of the CNJ206-TS analog complex has been proposed. This modelled structure differs from the crystallographically determined structure of the 17E8-**3** complex in that the anionic

phosphonate oxygens interact via H-bonding with two charge-neutral tyrosine hydroxyls rather than with a positively charged basic residue.

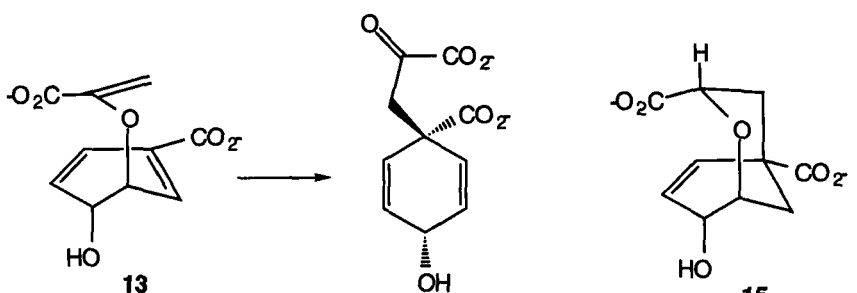


11



12

The 1F7 Chorismate Mutase System - Chorismate mutase is an enzyme that catalyzes the conversion of chorismate (13) to prephenate (14) which is the committed step in aromatic amino acid synthesis in bacteria, yeast, and higher plants (47). The reaction is a formal 2,3-sigmatropic rearrangement, or Claisen rearrangement, and the bicyclic TS analog 15 is a potent inhibitor of the enzyme (48,49). Catalytic antibodies have been generated using 15 in two separate laboratories and have been shown to have both similar and different mechanistic properties (50-53). The catalytic antibody 11F1-2E9 catalyzes the enantioselective conversion of 13 to 14 with a rate acceleration of 10^4, and facilitates catalysis by significantly lowering the entropy of activation ($\Delta S\ddagger$), presumably by using binding energy to orient the vinyl ether side chain of 13 in the reactive conformation (50,51). On the other hand, the independently generated catalytic antibody 1F7, displays a rate acceleration of 100 and catalysis by this antibody results entirely from lowering the enthalpy of activation ($\Delta H\ddagger$) compared to the uncatalyzed reaction (52,53). The entropy of activation for 1F7 catalysis is less favorable than the uncatalyzed reaction, and transfer NOE experiments indicate that 1F7 preferentially binds a high-energy diaxial conformer of 13 (54).



13 14 15

The crystal structure of the 1F7 Fab fragment complexed to 15 has been solved to 3.0 Å resolution (55). This structure can be compared directly with the crystal structure of the natural enzyme (monofunctional chorismate mutase from *Bacillus subtilis*) complexed to the same TS analog, 15 (56). Comparison of the structures shows that

both the catalytic antibody and natural enzyme contain an active site cleft that is complementary to TS analog **15**. Both proteins contain similar groups for binding the anionic non-bridgehead carboxylate; the interactions in 1F7 are from Arg^{H95} and a water molecule that bridges between the carboxylate and backbone amide of Asp^{H97}, whereas electrostatic interactions from the sidechain guanidinium groups of Arg^{90} and Arg^{7} are utilized in the enzyme. The active sites of the enzyme and 1F7 differ significantly in the make-up of side chain functional groups in the vicinity of the hydroxyl of **15**. The side chains of Glu^{78} and Cys^{75} interact with the hydroxyl group of **15** in the enzyme, and 1F7 has the amide side chain of Asn^{H33} interacting with this hydroxyl. This structural difference between the catalytic antibody and the natural enzyme (Figure 2) may be one of the main reasons that the activity of the catalytic antibody is 10^4 times lower than that of the enzyme and suggests that new TS analog designs that will elicit a carboxylate side chain in the vicinity of this hydroxyl could produce a more active catalyst.

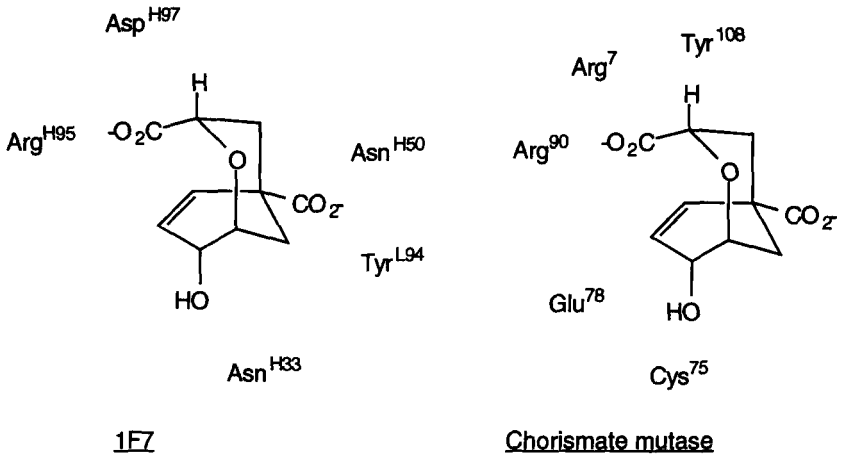


Figure 2: Comparison of the active site residues for 1F7 and Chorismate Mutase

NEW METHODS FOR OBTAINING CATALYTIC ANTIBODIES

A currently important research topic in the catalytic antibody field centers on the development of new methodology for identifying catalytic antibodies from the immune response. The traditional method involving hybridoma technology (9), basically the same method used to obtain monoclonal antibodies for diagnostics and probes, scores potential catalysts based on the indirect measure of TS-analog binding (16). Moreover, the process of hybridoma production results in significant B-cell attrition such that only a tiny fraction of the original B-cell population is converted to stable hybridoma cell lines. For example, a typical B-cell/myeloma fusion experiment starting with approximately 10^8 cells provides on the order of 10^3 stable hybridomas, meaning that only on the order of 0.001% of initial immunoglobulin population can be examined for catalytic activity at this stage. In practice, the 10^3 initially generated hybridomas typically yield about 100 TS analog-specific hybridomas and the cloning process reduces this number to a final panel of 10-50 monoclonal cell lines. Clearly improvement in the screening methodology is needed to increase the odds of identifying rare catalytic antibodies from the immunoglobulin repertoire that may have high levels of catalytic activity. Recently, several new methods that rely on the direct measure of catalytic activity rather than the indirect measure of TS analog binding have been reported, and these approaches have in some cases led to the discovery of more active catalytic antibodies.

Screening Hybridomas for Catalysis - A few different approaches for screening directly hybridoma supernatants for catalysis have been reported. The first such procedure reported, termed catELISA, relies on the use of an immobilized substrate bound to a microtiter plate and detection of the immobilized product with product-specific antibodies (57). This method has been used to identify a reasonably active esterolytic antibody (k_{cat} = 7.4 min^{-1}) from hybridoma supernatants resulting from phosphonate TS analog-immunized B-cells. In addition, a variation of this basic assay has been shown to be suitable for screening supernatants for antibodies that catalyze a bimolecular Diels-Alder reaction (58). In this case, the dienophile is immobilized to the solid support and the diene is present free in solution.

Reactions that liberate a chromogenic group are also in principle adaptable to screening for catalysis at the hybridoma supernatant stage. A successful example of this strategy is the identification of a highly active catalytic antibody that catalyzes a β-elimination reaction (59). The product of this reaction absorbs strongly at 340 nm, and this property was used to screen hybridoma supernatants prepared from B-cells immunized with a hapten designed to elicit a catalytic active site residue capable of functioning as a general base. Two highly active catalysts were identified from a population of >1000 hybridomas using this method and both catalysts have k_{cat} values of approximately 30 min^{-1} and rate accelerations of 10^8. In addition, the pH-rate profiles of both catalysts show a titratable catalytic residue with apparent pK_a of approximately 6. This pK_a value is reasonable for an active site Glu or Asp that would be anticipated from the hapten design.

Phage Libraries - For some applications, it has proven possible to bypass hybridoma production by cloning and expressing the immunoglobulin repertoire in bacteriophage libraries (60-71). Strategies utilizing the polymerase chain reaction (PCR) have been developed which make it possible to amplify selectively cDNA's encoding immunoglobulin light and heavy chains from B-cell mRNA (60,72). In addition, these methods are suitable for preparing both Fab and single chain Fv antibody fragments (73). Two potential drawbacks of this approach for identifying catalytic antibodies are the difficulties associated with expression of functional antibody fragments in bacteria, and the fact that in the cloning process, the light and heavy chain genes are randomly recombined such that the probability of pairing light and heavy chains in the same way as they were paired in the B-cells is very low. Nevertheless, successful results in screening phage libraries for catalytic antibodies have been reported (29).

A variation on this theme involves using filamentous phage-display (74)and *in vitro* selection to idenify catalytic antibodies that contain selectable active site catalytic residues. It has recently been reported that *in vitro* selection of an antibody library displayed on filamentous phage against an immobilized disulfide compound was successful in identifying catalytic antibodies that contain an active site cysteine capable of functioning as nucleophile in thioester hydrolysis (75). The cysteine-containing antibody was presumably selected on the basis of forming a covalent disulfide linkage with the immobilized disulfide compound *via* a disulfide exchange reaction. This strategy may in principle be generalized to other reaction types that are subject to nucleophilic catalysis.

References

C.A. Hasemann and J.D. Capra in "Immunoglobulins: Structure and Function," W. E. Paul, Ed., Raven Press, New York, 1989, p. 209.
M. Turner in "Molecules which Recognize Antigen," I. Roitt, J. Brostoff and D. Male, Ed., Gower Medical Publishing, New York, 1989, p. 5.
A. Fersht, "Enzyme Structure and Mechanism," Freeman, New York, 1985, p. 475.
L. Pauling, Chem. Eng. News, 24, 1375 (1946).

5. L. Pauling, Am. Sci., 36, 51 (1948).
6. A. Tramontano, K.D. Janda and R.A. Lerner, Science, 234, 1566 (1986).
7. S.J. Pollack, J.W. Jacobs and P.G. Schultz, Science, 234, 1570 (1986).
8. W.P. Jencks, "Catalysis in Chemistry and Enzymology," McGraw-Hill, New York, 1969, p. 228.
9. G. Kohler and C. Milstein, Nature, 256, 495 (1975).
10. R.A. Lerner, S.J. Benkovic and P.G. Schultz, Science, 252, 659 (1991).
11. P.G. Schultz, Science, 240, 426 (1988).
12. M.J. Powell and D.E. Hansen, Protein Eng., 3, 69 (1989).
13. B.S. Green and D.S. Tawfik, TIBTECH, 7, 304 (1989).
14. P.G. Schultz, Angew. Chem. Int. Ed. Engl., 28, 1283 (1989).
15. P.G. Schultz, Acc. Chem. Res., 22, 287 (1989).
16. K.M. Shokat and P.G. Schultz, Ann. Rev. Immunol., 8, 335 (1990).
17. P.G. Schultz, R.A. Lerner and S.J. Benkovic, Chem. Eng. News, 68, 26 (1990).
18. D. Hilvert and K.W. Hill, Meth. Enzymol., 203, 352 (1991).
19. M.C. Tedford and W.H. Stimson, Experentia, 47, 1129 (1991).
20. T.S. Scanlan and P.G. Schultz, Phil. Trans. R. Soc. Lond. B, 332, 157 (1991).
21. C.J. Suckling, Biochem. Soc. Trans., 216 (1991).
22. D. Hilvert, Pure and Appl. Chem., 64, 1103 (1992).
23. J.D. Stewart, L.J. Liotta and S.J. Benkovic, Acc. Chem. Res., 26, 396 (1993).
24. D. Hilvert, Acc. Chem. Res., 26, 552 (1993).
25. P.G. Schultz and R.A. Lerner, Acc. Chem. Res., 26, 391 (1993).
26. J.D. Stewart, J.F. Krebs, G. Siuzdak, A.J. Berdis, D.B. Smithrud and S.J. Benkovic, Proc. Nat. Acad. Sci.USA, 91, 7404 (1994).
27. G.M. Blackburn and P. Wentworth, Chem. Ind., 338 (1994).
28. D. Hilvert, Curr. Opin. Struct. Biol., 4, 612 (1994).
29. B. Posner, J. Smiley, I. Lee and S. Benkovic, Trends in Biochem. Sci., 19, 145 (1994).
30. K.D. Janda, Pure and Appl. Chem., 66, 703 (1994).
31. J. Guo, W. Huang and T.S. Scanlan, J. Am. Chem. Soc., 116, 6062 (1994).
32. K.M. Shokat, M.K. Ko, T.S. Scanlan, L. Kochersperger, S. Yonkovich, S. Thaisrivongs and P.G. Schultz, Angew. Chem. Int. Ed. Engl., 29, 1296 (1990).
33. H. Suga, O. Ersoy, T. Tsumuraya, J. Lee, A.J. Sinskey and S. Masamune, J. Am. Chem. Soc., 116, 487 (1994).
34. H. Suga, O. Ersoy, S.F. Williams, T. Tsumuraya, M. Margolies, A.J. Sinskey and S. Masamune, J. Am. Chem. Soc., 116, 6025 (1994).
35. K.D. Janda, D. Schloeder, S.J. Benkovic and R.A. Lerner, Science, 241, 1188 (1988).
36. B.L. Iverson and R.A. Lerner, Science, 243, 1184 (1989).
37. M.T. Martin, T.S. Angeles, R. Sugasawara, N.I. Aman, A.D. Napper, M.J. Darsley, R.I. Sanchez, P. Booth and R.C. Titmas, J. Am. Chem. Soc., 116, 6508 (1994).
38. E. Baldwin and P.G. Schultz, Science, 245, 1104 (1989).
39. D.Y. Jackson, J.R. Prudent, E.P. Baldwin and P.G. Schultz, Proc. Natl. Acad. Sci. USA, 88, 58 (1990).
40. J.D. Stewart, V.A. Roberts, N.R. Thomas, E.D. Getzoff and S.J. Benkovic, Biochemistry, 33, 1994 (1994).
41. V.A. Roberts, J. Stewart, S.J. Benkovic and E.D. Getzoff, J. Mol. Biol., 235, 1098 (1994).
42. G.W. Zhou, J. Guo, W. Huang, R.J. Fletterick and T.S. Scanlan, Science, 265, 1059 (1994).
43. J. Kraut, Annu. Rev. Biochem., 46, 331 (1977).
44. J. Guo, W. Huang, G.W. Zhou, R.J. Fletterick and T.S. Scanlan, Proc. Natl. Acad. Sci. USA, 92, in press (1995).
45. D.S. Tawfik, R.R. Zemel, R. Arad-Yellin, B.S. Green and Z. Eshhar, Biochemistry, 29, 9916 (1990).
46. B. Golinelli-Pimpaneau, B. Gigant, T. Bizebard, J. Navaza, P. Saludjian, R. Zemel, D.S. Tawfik, Z. Eshhar, B.S. Green and M. Knossow, Structure, 2, 175 (1994).
47. U. Weiss and J.M. Edwards, "The Biosynthesis of Aromatic Amino Acid Compounds," Wiley, New York, 1980, p. 134.
48. P.A. Bartlett and C.R. Johnson, J. Am. Chem. Soc., 107, 7792 (1985).
49. P.A. Bartlett, Y. Nakagawa, C.R. Johnson, S.H. Reich and A.J. Luis, J. Org. Chem., 53, 3195 (1988).
50. D.Y. Jackson, J.W. Jacobs, S. Reich, R. Sugasawara, P.A. Bartlett and P.G. Schultz, J. Am. Chem. Soc., 110, 4841 (1988).
51. D.Y. Jackson, M.N. Liang and P.G. Schultz, Angew. Chem. Int. Ed. Eng., 31, 182 (1992).
52. D. Hilvert, S.H. Carpenter, K.D. Nared and M.-T.M. Auditor, Proc. Natl. Acad. Sci. USA, 85, 4953 (1988).
53. D. Hilvert and K.D. Nared, J. Am. Chem. Soc., 110, 5593 (1988).
54. A.P. Campbell, T.M. Tarasow, W. Massefski, P.E. Wright and D. Hilvert, Proc. Natl. Acad. Sci. USA, 90, 8663 (1993).
55. M.R. Haynes, E.A. Stura, D. Hilvert and I.A. Wilson, Science, 263, 646 (1994).

56. Y.M. Chook, H. Ke and W.N. Lipscomb, Proc. Natl. Acad. Sci. USA, 90, 8600 (1993).
57. D.S. Tawfik, B.S. Green, R. Chap, M. Sela and Z. Eshhar, Proc. Natl. Acad. Sci. USA, 90, 373 (1993).
58. G. MacBeath and D. Hilvert, J. Am. Chem. Soc., 116, 6101 (1994).
59 S.N. Thorn, R.G. Daniels, M.-T.M. Auditor and D. Hilvert, Nature, 373, 228 (1995).
60. W.D. Huse, L. Sastry, S.A. Iverson, A.S. Kang, M. Alting-Mees, D.R. Burton, S.J. Benkovic and R.A. Lerner, Science, 246, 1275 (1989).
61. J. McCafferty, A.D. Griffiths, G. Winter and D.J. Chiswell, Nature, 348, 552 (1990).
62. A.S. Kang, C.F. Barbas, K.D. Janda, S.J. Benkovic and R.A. Lerner, Proc. Natl. Acad. Sci. USA, 88, 4363 (1991).
63. J.D. Marks, H.R. Hoogenboom, T.P. Bonnert, J. McCafferty, A.D. Griffiths and G. Winter, J. Mol. Biol., 222, 581 (1991).
64. C.F. Barbas III, A.S. Kang, R.A. Lerner and S.J. Benkovic, Proc. Natl. Acad. Sci. USA, 88, 7978 (1991).
65. C.F. Barbas III, J.D. Bain, D.M. Hoekstra and R.A. Lerner, Proc. Natl. Acad. Sci. USA, 89, 4457 (1992).
66. R.A. Lerner, A.S. Kang, J.D. Bain, D.R. Burton and C.F. Barbas III, Science, 258, 1313 (1992).
67. H. Gram, L.A. Marconi, C.F. Barbas III, T.A. Collet, R.A. Lerner and A.S. Kang, Proc. Natl. Acad. Sci. USA, 89, 3576 (1992).
68. T.A. Collet, P. Roben, R. O'Kennedy, C.F. Barbas III, D.R. Burton and R.A. Lerner, Proc. Natl. Acad. Sci. USA, 89, 10026 (1992).
69. C.F. Barbas III, J.S. Rosenblum and R.A. Lerner, Proc. Natl. Acad. Sci. USA, 90, 6385 (1993).
70. C.F. Barbas III, W. Amberg, A. Simoncsits, T.M. Jones and R.A. Lerner, Gene, 137, 57 (1993).
71. A.D. Griffiths, S.C. Williams, O. Hartley, I.M. Tomlinson, P. Waterhouse, W.L. Crosby, R.E. Kontermann, P.T. Jones, N.M. Low, T.J. Allison, T.D. Prospero, H.R. Hoogenboom, A. Nissim, J.P.L. Cox, J.L. Harrison, M. Zaccolo, E. Gherardi and G. Winter, EMBO J., 13, 3245 (1994).
72. H.R. Hoogenboom, A.D. Griffiths, K.S. Johnson, D.J. Chiswell, P. Hudson and G. Winter, Nucl. Acids Res., 19, 4133 (1991).
73. G. Winter and C. Milstein, Nature, 349, 293 (1991).
74. G.P. Smith, Science, 228, 13151317 (1985).
75. K.M. Janda, C.-H.L. Lo, T. Li, C.F. Barbas III, P. Wirsching and R.A. Lerner, Proc. Natl. Acad. Sci. USA, 91, 2532 (1994).

Chapter 28. Structure-Based Design from Flexible Ligands

Robert S. McDowell and Dean R. Artis
Genentech, Inc.
South San Francisco, CA 94080

Introduction - Significant recent progress has been made in designing lead compounds based on the structures of target proteins (typically enzymes) complexed with peptide or non-peptide inhibitors (1,2). While the ultimate therapeutic utility of many of these compounds remains to be determined, the diversity of molecules generated by structure-based methods is quite impressive. Often, of course, the structure of the target is not known and no small molecule lead is available. Instead, the medicinal chemist has as a starting point a flexible molecule (frequently a peptide) with the desired *in vitro* profile, but which lacks the pharmacological attributes required for a medicinal lead. Traditionally, such molecules are sequentially modified to evolve a lead series (3). However, leads derived from this process often maintain the topology of the original molecule. Given sufficient information, topographical or structure-based approaches can provide a rationale for the synthesis of molecules with dramatically different topologies. This chapter summarizes some strategies that have been used to obtain structural information from highly flexible molecules. While these strategies may not yield a model that has the atomic level of detail afforded by X-ray crystallography, examples will illustrate that even approximate structural models can provide a useful springboard for the discovery of novel lead series. Although the discussion will focus on peptides, it is clear that similar strategies can be applied to other compounds that can be assembled in a modular fashion, including hits obtained from combinatorial synthesis.

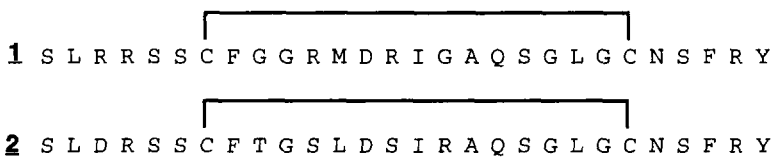
Cyclic Peptides - The bound structure of a peptide, whether obtained by X-ray crystallography or by NMR (4), provides the most convincing blueprint for a design effort. In a majority of cases, however, it is necessary to rely on the structure - if any - of the ligand itself. Because peptide conformation is largely determined by nonbonded interactions and environmental (particularly solvent) effects, NMR provides the most direct means of observing peptide structure and dynamics (5). Cyclosporin-A, which adopts a different conformation in chloroform than when it is bound to cyclophilin (6,7), provides a cautionary note regarding over interpretation of the free-solution structures of peptides, particularly if those structures cannot be determined in a biologically relevant solvent system.

Significant enhancements in activity are often observed as a result of cyclizing the functionally important residues of a peptide. Efforts to design cyclic peptides that impart a particular structure are frustrated by the dependence of peptide conformation on noncovalent interactions that are not reliably reproduced by current forcefield approaches. Additionally, few robust cyclic peptide frameworks have been identified which maintain backbone conformation independent of sidechain composition. Exceptions include the cyclic hexapeptides of the form (cyclo) X-X-Gly-X-X-Gly, which often adopt extended structures consisting of β-turns initiated at Gly^3 and Gly^6 (8), and cyclic pentapeptides containing a single D-amino acid, which maintain a combination of β– and γ-turns (9).

Because of these limitations, most peptides are optimized primarily on the basis of their activities, leaving the chemist to ascertain whether their solution structures are biologically relevant. Parallels between solution structure and biological activity

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

have been observed for a number of cyclic peptides. A recent example illustrates the potential power of biological selection to optimize both structure and activity. The 28-residue atrial natriuretic peptide (ANP) hormone is involved in regulation of blood pressure, fluid, and electrolyte homeostasis (10). One of the 3 known natriuretic peptide receptors (NPR), NPR-A (11) appears to be responsible for most of the biological effects of ANP (12), while a different receptor (NPR-C) is thought to reduce the serum half-life of ANP by promoting internalization and degradation (13). Wild-type ANP (**1**) has a comparable affinity (1-2 pM) for NPR-A and NPR-C, but has little discernible structure in either water or DMSO (14). Monovalent phage display technology was used to develop a variant of ANP (**2**) that retains wild-type activity at NPR-A, but with 10^5-fold lower affinity for NPR-C (15). Unlike the wild-type ANP, (**2**) was sufficiently ordered in water to permit an NMR structure to be determined (16). While it is unknown how this structure relates to the bound conformation of ANP, it is provocative that selection for binding specificity also resulted in selection for structure. Importantly, the presence of a structure allows for reductionist strategies to be implemented to determine whether the critical interactions of ANP with NPR-A can be accommodated in a smaller molecule.

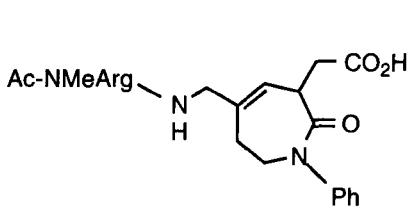


Somatostatin represents a successful example of such a strategy, in which the native tetradecapeptide was systematically reduced to a constrained cyclic hexapeptide based on a proposed bioactive conformation (17). Following the pioneering work of Freidinger and Veber, potent, structured peptide analogs of somatostatin have been developed, all of which possess a characteristic β-turn conformation of the critical Phe-Trp-Lys-Thr quartet (18,19). The biological activity of somatostatin is clearly localized in a compact set of interactions for which a well-defined structural model is available. It is surprising, therefore, that no highly active nonpeptide analogs of somatostatin have been reported, although a scaffold designed from the hexapeptide structure showed some signs of activity (20).

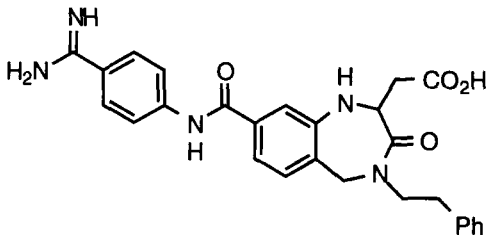
By contrast, the arginine-glycine-aspartic acid (RGD) sequence has provided fertile ground for both the construction of potent, structured peptides and for the subsequent *de novo* design of nonpeptide lead structures. Peptides containing the RGD sequence are able to successfully inhibit the binding of fibrinogen to platelet glycoprotein IIb/IIIa (GPIIb/IIIa), and thus serve as effective inhibitors of platelet aggregation (21). NMR studies of the active cyclic disulfide (2-mercaptobenzoyl)-N$^{\alpha}$-methylArg-Gly-Asp-2-mercaptoanilide indicated the presence of two potential conformations in solution, one of which was also observed crystallographically (22). This structure featured an extended RGD sequence with a C7 conformation of the aspartic acid residue. A nonpeptide mimic (**3**) designed to replace the C7 aspartic acid (23) served as the nucleus of a highly active lead compound (**4**) that matched the topography of the RGD sequence (24).

An alternate cyclic peptide series based on cyclo-[S-Acetyl-DTyr-Arg-Gly-Asp-Cys]-OH (25) provided a very different design template. Modifying the stereochemistries of both the thioether linkage and the amino acid preceding the RGD sequence produced variations in activity that paralleled the degree of rigidity observed by NMR (26). The most active molecule had an unambiguous structure in water which featured a cupped conformation of the RGD sequence. This structure was used to

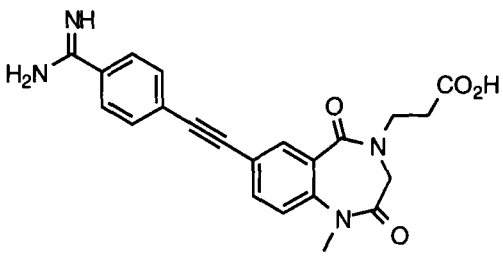
design a potent nonpeptide scaffold (5) that enforced the observed shape of the RGD epitope without reproducing the local backbone conformation of any specific residue (27). It is remarkable that these different design strategies, which started from active structured peptides with dissimilar RGD conformations, converged on topologically similar (though topographically distinct) molecules.



3



4



5

Local Constraints - Using modified or unnatural amino acids further expands the repertoire of tools available for manipulating peptide structure. Recent reviews provide excellent coverage of this field (28-30). While it is not the aim of this work to inventory the large number of amino acid surrogates that have been constructed, a representative set of examples will be discussed in the context of their contributions to mapping peptide conformations. Generally, work has focused in two areas: replacements or modifications to the peptide backbone, and covalently constrained sidechain analogs. The two are not necessarily independent, and are certainly intertwined from the perspective of conformational analysis.

Many modifications of the backbone amide bond (-CO-NH-) were originally implemented to decrease metabolism by proteases and improve the therapeutic potential of peptides(3). The systems developed for these purposes can also be informative in probing conformational issues and exploring peptide SAR. The replacements can retain (-HC=CH-, -HN-CO-) or increase (-CH_2-X-) the level of flexibility at the inter-residue linkage. Simple N-alkylation can be an extremely useful modification, in that it simultaneously changes the hydrogen-bonding capabilities of the amide bond and strongly biases the conformation of the preceding residue in the chain (31). Changes at the α-carbon, such as α,α–disubstitution (32) or use of dehydro-amino acid residues (33) impart further conformational preferences. Sidechain modifications include methyl-substitutions (e.g. penicillamine or o,o'-dimethyl tyrosine), or homologation (norleucine, phenylglycine, homophenylalanine).

Residues such as 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) and 2-amino-2-indane carboxylic acid constrain both backbone and sidechain degrees of freedom.

Conformational constraint is often imposed by using covalent linkages to enforce a set of torsional preferences. However, combining selected conventional residues can induce steric effects that result in a strong bias for certain local motifs. A simple example is the well-known DPro/LPro β-turn (34). Various approaches have been taken to induce canonical secondary structure with small, structured, organic nucleating groups. Kemp and coworkers have successfully induced the formation of α-helical peptides (35,36). Recent reports have focused on the propensity of one system to nucleate β-sheet structure in medium sized peptides (37,38). Replacements for groups of residues have also been also used to enforce portions of β-turns (39) and γ-turns (40). A number of reviews have concentrated on the use of these systems in mimetics of bioactive peptides (28,39,41,42). The C7 turn replacement for the RGD aspartic acid residue (**3**) represents a particularly compelling example, in that it served as a precursor to a useful nonpeptide scaffold. Given that many peptides may bind in a conformation not well represented by any canonical structure, we expect much more effort in the future to be given to the more general design of "spacing groups" for peptide substructures. For example, a rigid cyclization linkage constructed in an effort to further enforce the extended RGD conformation observed in the cyclic disulfides (43) led to a compound that was highly active and that unambiguously displayed the desired conformation in solution (44).

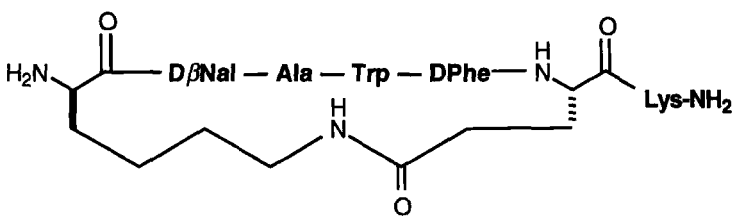
The tools discussed above have been used successfully to develop structural models of active peptides through an iterative cycle of hypothesis, experimental evaluation, and reassessment of hypothesis. Angiotensin II (AII), a vasoactive octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), provides an example of this approach. AII has been studied under a variety of conditions and many different types of secondary structure have been proposed in connection with the molecule (45). Simple cyclizations of the N- and C-terminal residues produced inactive or weakly active molecules (46,47). Amide bond replacements generally did not prove fruitful (48). Efforts over many years in Marshall's group (49) and others (50) found that α,α-disubstituted residues were tolerated at several positions, and that constraining residues like proline had modest to significant consequences at positions 3 and 5 (51), implying the presence of an ill-defined set of turns through the middle of the peptide. Although deleting the first and second residues resulted in significant decreases in potency, the first three residues could be replaced by alkoyl amines of varying lengths with a modest decrease in activity (52). Truncating one or two of the C-terminal residues gave either inactive compounds or moderately active antagonists (53). The sensitivity of the C-terminus to modification was underscored by two points. Changing the terminal acid to an alcohol resulted in a 10^5-fold decrease in potency (54), and potent antagonists could be obtained simply by inverting the Phe^8 stereocenter (55), or by replacing Phe^8 with L-residues containing alkyl sidechains (56). Additionally, while the cyclic [$Hcys^3$-$Hcys^5$]-AII analog (Hcys = homocysteine) was shown to be equipotent with AII with the C-terminal SAR retained (57), smaller cycles (e.g. Cys^3-Cys^5) were less active. The bicyclic [$Hcys^3$-Mpt^5]-AII also retained full potency (Mpt = 4-mercaptoproline-trans, which functions as a combined homocysteine and proline analog), but other 3,5-disubstituted analogs again had increasingly reduced activity (58), reflecting the enforcement of inappropriate conformational constraints. Recent NMR studies have revealed that the cyclized portion of [$Hcys^3$-Mpt^5]-AII has a well-defined structure (59). Based on the data above, one might now envision the molecule schematically as a central scaffold presenting an alkyl amine to one side and a constrained C-terminal tripeptide to the other.

Another peptide hormone, bradykinin (BK, Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) has also been intensively studied. There is evidence for a C-terminal β–turn (centered on the Pro-Phe residues) of the peptide (60) and related antagonists (61,62), though the relative disposition of the N-terminus is uncertain. Systematic modification of the peptide has resulted in both agonists and antagonists of enhanced potency. The majority of amide bond replacements and conformational adjustments to backbone and sidechain atoms have not improved activity, and the main classes of antagonists have been derived from [DPhe^7]-BK, a molecule of only modest potency (63). The C-terminal β-turn in some of these antagonists has been induced using DTic-Oic (Oic = (S,S,S)-octahydroindole-2-carboxylic acid) (64), leading to a group of molecules typified by the compound HOE-140 (DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DTic-Oic-Arg, Hyp = L-4-hydroxy-L-proline; Thi = β–(2-thienyl)-L-alanine) (65). Full length cyclic analogs of a related compound, (DArg-Arg-Pro-Hyp-Gly-Phe-Ser-DTic-Oic-Arg) were developed using an approach of N-methylation and Cα–methylation at the Gly and Phe residues (66). The most potent of the resulting compounds, cyclo-DArg-Arg-[Cys-Pro-Gly-Cys]-Ser-DTic-Oic-Arg, had a K_i = 1.5 nM, down about 20-fold from the parent molecule. A recent report has indicated that the cyclic substructure in this compound can be replaced with a flexible alkoyl amine linker, yielding antagonists of the form (DArg-Arg-xxx-Ser-DTic-Oic-Arg, where xxx represents the linker), that range in activity from 471 nM to 32 nM as the number of methylenes in the linker increases from 4 to 12 (67). Further reduction of the antagonists was enabled by the discovery that the residues of the C-terminal β-turn could be incorporated into a cyclic hexapeptide, yielding smaller analogs of reasonable potency (68). The most active simple analog, cyclo-[DLys-Phe-Ser-DTic-Oic-Arg], had a K_d = 840 nM; substitution on the lysine sidechain gave some improvement. The presence of the β-turn centered on DTic-Oic was confirmed via NMR spectroscopy. By contrast, a less potent analog containing Pro-Phe in these positions was found to exist in two conformations, neither of which contained the analogous β–turn. Thus, the bias toward the "preferred" conformation resulted in an improvement in potency in these interesting systems. The agonists and antagonists discussed above have similarity both in sequence and in the SAR resulting from certain residue replacements. Surprisingly, however, recent receptor mutagenesis and binding experiments using BK and HOE-140 as ligands for the BK receptor reveal that these peptides bind to largely different portions of the receptor (69). These findings underscore the importance of characterizing the site of ligand binding while developing structure-activity hypotheses.

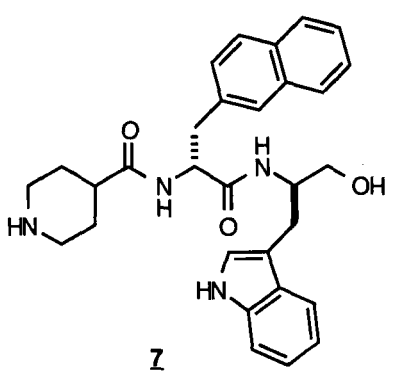
Indirect Approaches - Given the limitations of forcefield methods, it is rarely possible to reliably determine the active conformation of a highly flexible molecule in the absence of additional restraints. Useful procedures do exist, however, for exploring energetically accessible conformations available to a molecule or group of molecules. Systematic conformation searching (70,71), distance geometry (72,73), molecular dynamics, and Monte Carlo methods (74,75) sample the conformations of individual compounds. Conformations that overlap corresponding groups from a collection of active analogs can be subsequently identified by a number of techniques based on atomic superimposition, inter atomic distances, or induced molecular fields (76). Ensemble distance geometry (77) and ensemble molecular dynamics (78) procedures enforce such an overlap *a priori* by identifying only those conformations of a set of molecules that superimpose important shared groups. The practical utility of any of these methods is determined primarily by the diversity of the starting structures: compounds with very similar flexible backbones probably share many biologically irrelevant alignments, while dissimilar molecules are likely to have a limited number of potential consensus structures.

This approach of re-scaffolding the backbone is ideally suited to ligands whose biological activity is mediated by sidechain interactions. Growth hormone releasing

peptides (GHRPs) (79,80), derived from the C-terminus of Met-enkephalin, promote growth hormone release from pituitary somatotrophs and show promise as agents for treating certain disease states associated with growth hormone deficiency. The secretagogue effects of the prototype GHRP-6 peptide, His-DTrp-Ala-Trp-DPhe-Lys-NH_2, result primarily from the sidechains of the charged N-terminal histidine and the central aromatic residues (81). The spatial dispositions of these critical sidechains were explored by constructing an alternate peptide backbone, His-DTrp-DTrp-Phe-Lys-NH_2, which was derived from an early progenitor of GHRP (82). Analogous modifications of the two peptide series produced parallel enhancements in activity, suggesting that the bioactive conformations of these structurally distinct secretagogues confer a similar three-dimensional presentation of the key aromatic residues. Ensemble dynamics studies of the two classes of molecules indicated that only two consensus conformations of the aromatic cores were energetically possible. In parallel, a highly potent cyclic analog of GHRP-6 was developed (**6**, DβNal = D-betanaphthylalanine) which was found to be structured in water. The solution structure of this analog was nearly identical to one of the consensus conformations identified by the dynamics procedure. These findings suggest that it is possible to obtain a useful structural model by arraying a set of pharamacophoric elements on different flexible backbones, using a "triangulation strategy" to determine shared three-dimensional presentations. More importantly, this alternate scaffold was systematically reduced to a low molecular weight secretagogue (**7**) with high *in vivo* activity (82).



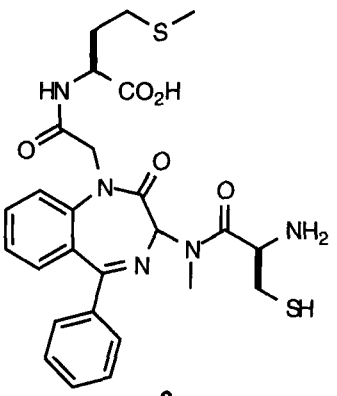
6



7

A recently developed inhibitor of the Ras farnesyltransferase enzyme provides an additional example in which a useful lead compound was discovered in an attempt to explore structure. The transforming activity of the oncogenic mutant Ras protein requires the attachment of a farnesyl group to an unpaired cysteine near the C-terminus (83-85). This attachment is catalyzed by the farnesyltransferase enzyme, which can be inhibited by tetrapeptides based on the Cys-A1-A2-X motif found at the C-termini of Ras and other prenylated substrates (86). A turn-like conformation of this

motif was proposed based on circumstantial evidence from peptide studies (87). Replacing the central hydrophobic residues with a number of rigid turn mimetics led to the discovery of an inhibitor (**8**) that, unlike the initial peptide, successfully reverted the phenotype of Ras-transformed cells (88).



8

Discussion - It is clear from the examples mentioned above that many powerful tools exist for deducing putative bioactive conformations of flexible molecules. The biological importance of the Phe-Trp-Lys-Thr turn in somatostatin is underscored by the many potent, structured peptide analogs that have been developed. Successive rigidification of the RGD epitope in cyclic peptides has led in two cases to models that directed the design of novel nonpeptide lead compounds. Angiotensin II and bradykinin illustrate how local conformational constraints can be combined with cyclization to sequentially develop high-confidence structural models. Excepting the GPIIb/IIIa antagonists, however, few compelling examples exist in which these types of models have been used successfully to design fundamentally new chemical entities. Certainly, no reliable flowchart exists to guide the chemist in designing novel compounds based purely on a structural model, while the challenges inherent in any medicinal chemistry program still remain. Similar molecules that share a good overlap on a computer screen may be found to bind in completely different ways when co-crystallized with the target protein (89). Pharmaceutical company stockrooms undoubtedly contain numerous compounds that structurally resemble a well-characterized small molecule lead, but which completely lack activity for unknown reasons. A structural model may also lack the resolution required to precisely identify the positions of key functional groups, thereby frustrating attempts to use database methods to search for suitable design templates. Given these challenges and the historical success of toplogically-driven discovery strategies, it is not surprising that structure-based approaches are often considered too speculative to be of practical use in the pharmaceutical industry.

Nonetheless, this speculation has clearly yielded some successes. The nonpeptide GPIIb/IIIa antagonists described above are so topologically distinct from the original cyclic peptides that they probably would not have been discovered by progressively modifying the RGD sequence. The alternate backbone constructed to map the spatial arrangements of the GHRP aromatic core served not only as a useful conformational probe, but also as a novel starting point for a classical optimization strategy. Testing a hypothetical structural model of the Cys-A1-A2-X motif helped to catalyze the discovery of a novel lead compound. As a discovery strategy, using structural approaches to new lead generation can clearly yield diverse classes of compounds. The benefits of such diversity from both pharmacological and intellectual property perspectives are obvious. Structural models of highly flexible molecules are

often "incomplete" and evolve dynamically through interdisciplinary collaboration. Nonetheless, even an imprecise or speculative model more than serves its purpose if it can help define hypotheses that stimulate novel medicinal chemistry approaches to the discovery of new chemical entities.

REFERENCES

1. P. M. Colman, Curr. Op. Struct. Biol., 4, 868 (1994).
2. I. D. Kuntz, E. C. Meng and B. K. Shoichet, Acc. Chem. Res., 27, 117 (1994).
3. A. S. Dutta, Adv. Drug Res., 21, 145 (1991).
4. S. W. Fesik, J. Biomol. NMR, 3, 261 (1993).
5. O. Jardetzky and J. F. Lefevre, FEBS-Lett., 338, 246 (1994).
6. S. W. Fesik, R. T. Gampe Jr., H. L. Eaton, G. Gemmecker, E. T. Olejniczak, P. Neri, T. F. Holzman, D. A. Eagan, R. Edalji, R. Helfrich, J. Hochlowski and M. Jackson, Biochemistry, 30, 6574 (1991).
7. C. Weber, G. Wider, B. vonFreyberg, R. Traber, W. Braun, H. Widmer and K. Wüthrich, Biochemistry, 30, 6574 (1991).
8. G. D. Rose and L. M. Gierasch, J. A. Smith, Adv. Prot. Chem., 37, 1 (1985).
9. H. Kessler, in "Trends in Drug Research," V. Claasen, Ed., Elsevier Science Publishers, Amsterdam, 1990, p. 73.
10. A. Rosenzweig and C. E. Seidman, Ann. Rev. Biochem., 60, 229 (1991).
11. D. G. Lowe, M.-S. Chang, R. Hellmiss, E. Chen, S. Singh, D. L. Garbers and D. V. Goeddel, EMBO J., 8, 1377 (1989).
12. T. Maack, Annu. Rev. Physiol., 54, 11 (1992).
13. F. Fuller, J. G. Porter, A. Arfsten, J. Miller, J. W. Schilling, R. M. Scarborough, J. A. Lewicki and D. B. Schenk, J. Biol. Chem., 263, 9395 (1988).
14. S. Koyama, Y. Kobayashi, T. Ohkubo, Y. Kyogoku, A. Sato, M. Kobayashi and N. Go, Protein Eng., 3, 393 (1990).
15. B. C. Cunningham, D. G. Lowe, B. Li, B. D. Bennett and J. A. Wells, EMBO J., 13, 2508 (1994).
16. W. J. Fairbrother, R. S. McDowell and B. C. Cunningham, Biochemistry, 33, 8897 (1994).
17. R. M. Freidinger and D. F. Veber, in "Conformationally Directed Drug Design" ACS Monograph Series # 251 J. A. Vida, M. Gordon, Eds., American Chemical Society, Washington, D.C., 1984, p. 169.
18. H. Kessler, J. W. Bats, C. Griesinger, S. Koll, M. Will and K. Wagner, J. Am. Chem. Soc., 110, 1033 (1988).
19. G. VanBinst and D. Wourwe, Peptide Res., 5, 8 (1992).
20. K. C. Nicolaou, J. M. Salvino, K. Raynor, S. Pietranico, T. Reisine, R. M. Freidinger and R. Hirschmann, Pept. Chem. Struct. Biol. Proc. Am. Pept. Symp. (1989), 11, 881 (1990).
21. E. F. Plow and M. H. Ginsberg, Prog. Hemost. Thromb., 9, 117 (1989).
22. K. D. Kopple, P. W. Baures, J. W. Bean, C. A. D'Ambrosio, J. L. Hughes, C. E. Peishoff and D. S. Eggleston, J. Am. Chem. Soc., 114, 9615 (1992).
23. J. F. Callahan, J. W. Bean, J. L. Burgess, D. S. Eggleston, S. M. Hwang, K. D. Kopple, P. F. Koster, A. Nichols, C. E. Peishoff, J. M. Samanen, J. A. Vasko, A. Wong and W. F. Huffman, J. Med. Chem., 35, 3970 (1992).
24. T. W. Ku, F. E. Ali, L. S. Barton, J. W. Bean, W. E. Bondinell, J. L. Burgess, J. F. Callahan, P. R. Calvo, L. Chen, D. S. Eggleston, J. G. Gleason, W. F. Huffman, S. M. Hwang, D. R. Jakas, C. B. Karash, R. M. Keenan, K. D. Kopple, W. H. Miller, K. A. Newlander, A. Nichols, M. F. Parker, C. E. Peishoff, J. M. Samanen, I. Uzinskas and J. W. Venslavsky, J. Am. Chem. Soc., 115, 8861 (1994).
25. P. L. Barker, S. Bullens, S. Bunting, D. J. Burdick, K. S. Chan, T. Deisher, C. Eigenbrot, T. R. Gadek, R. Gantzos, M. T. Lipari, C. D. Muir, M. A. Napier, R. M. Pitti, A. Padua, C. Quan, M. Stanley, M. Struble, J. Y. K. Tom and J. P. Burnier, J. Med. Chem., 35, 2040 (1992).
26. R. S. McDowell and T. R. Gadek, J. Am. Chem. Soc., 114, 9245 (1992).
27. R. S. McDowell, B. K. Blackburn, T. R. Gadek, L. R. McGee, T. Rawson, M. E. Reynolds, K. D. Robarge, T. C. Somers, E. D. Thorsett, M. Tischler, R. R. Webb III and M. C. Venuti, J. Am. Chem. Soc., 116, 5077 (1994).
28. B. A. Morgan and J. A. Gainor, Annu. Rep. Med. Chem., 24, 243 (1989).
29. J. Rizo and L. M. Gierasch, Annu. Rev. Biochem., 61, 387 (1992).
30. G. R. Marshall, Tetrahedron, 49, 3547 (1993).
31. G. R. Marshall and H. E. Bosshard, Circ. Res., 31 (Suppl. 2), II-143 (1972).
32. G. R. Marshall, E. E. Hodgkin, D. A. Langs, G. D. Smith, J. Zabrocki and M. T. Leplawpy, Proc. Natl. Acad. Sci. USA, 87, 487 (1990).
33. A. C. Bach and L. M. Gierasch, Biopolymers, 25, S175 (1986).
34. J. W. Bean, K. D. Kopple and C. E. Peishoff, J. Am. Chem. Soc., 114, 5328 (1992).

35. D. S. Kemp, T. P. Curran, W. M. Davis, J. G. Boyd and C. Muendel, J. Org. Chem., 56, 6672 (1991).
36. D. S. Kemp, T. P. Curran, J. G. Boyd and T. J. Allen, J. Org. Chem., 56, 6683 (1991).
37. H. Diaz, K. Y. Tsang, D. Choo and J. W. Kelly, Tetrahedron, 49, 3533 (1993).
38. J. P. Schneider and J. W. Kelly, J. Am. Chem. Soc., 117, 2533 (1995).
39. J. B. Ball and P. F. Alewood, J. Mol. Recognit., 3, 55 (1990).
40. D. S. Kemp and J. S. Carter, J. Org. Chem., 54, 109 (1989).
41. G. Holzemann, Kontakte (Darmstadt), 1, 3 (1991).
42. Symposium in Print : BioMed. Chem. Lett., 3, 797ff (1993).
43. S. Jackson, W. DeGrado, A. Dwivedi, A. Parthasarathy, A. Higley, J. Krywko, A. Rockwell, J. Markwalder, G. Wells, R. Wexler, S. Mousa and R. Harlow, J. Am. Chem. Soc., 116, 3220 (1994).
44. A. C. Bach II, C. J. Eyermann, J. D. Gross, M. J. Bower, R. L. Harlow, P. C. Weber and W. F. DeGrado, J. Am. Chem. Soc., 116, 3207 (1994).
45. J. V. Duncia, A. T. Chiu, D. J. Carini, G. B. Gregory, A. L. Johnson, W. A. Price, G. J. Wells, P. C. Wong, J. C. Calabrese and P. B. M. W. M. Timmermans, J. Med. Chem, 33, 1312 (1990).
46. J. M. Matsoukas, M. N. Scanlon and G. J. Moore, J. Med. Chem., 27, 404 (1984).
47. J. L. DeCoen, E. Ralson, J. P. Durieux and A. Loffet, in "Peptides: Chemistry, Structure and Biology," R. Walter, J. Meinhofer, Eds., Ann Arbor Science, Ann Arbor MI, 1975, p.553.
48. R. Mohan, Y.-L. Chou, R. Bihovsky, J. W. C. Lumma, P. W. Erhardt and K. J. Shaw, J. Med. Chem., 34, 2402 (1991).
49. J. Turk and P. Needleman, G. R. Marshall, Mol. Pharmacol., 12, 217 (1976).
50. M. C. Khosla, K. Stachowiak, R. R. Smelby, F. M. Bumpus, F. Piriou, K. Lintner and S. Fermandjan, Proc. Nat. Acad. Sci. USA, 78, 757 (1981).
51. J. Samanen, T. Cash, D. Narindray, E. Brandeis, W. Adams, H. Weideman, T. Yellin and D. Regoli, J. Med. Chem., 34, 3036 (1991).
52. P. R. Bovy, J. M. O'Neal, E. McMahon, M. Palomo, G. J. Smits, A. J. Trapani and D. McGraw, Eur. J. Med. Chem., 25, 589 (1990).
53. P. R. Bovy, J. M. O'Neal, G. M. Olins, D. R. Patton, E. G. McMahon, M. Palomo, J. P. Koepke, K. S. Salles, A. J. Trapani, G. J. Smits, D. E. McGraw and W. C. Hutton, J. Med. Chem., 33, 1477 (1990).
54. K.-h. Hsieh and G. R. Marshall, J. Med. Chem., 29, 1969 (1986).
55. J. Samanen, D. Narindray, J. W. Adams, T. Cash, T. Yellin and D. Regoli, J. Med. Chem., 31, 510 (1988).
56. A. Aumelas, C. Sakarellos, K. Lintner, S. Fermandjian, M. C. Khosla, R. R. Smeby and F. M. Bumpus, Proc. Nat. Acad. Sci. USA, 82, 1881 (1985).
57. K. L. Spear, M. S. Brown, E. J. Reinhard, E. G. McMahon, G. M. Olins, M. A. Palomo and D. R. Patton, J. Med. Chem., 33, 1935 (1990).
58. K. Plucinska, T. Kataoka, M. Yodo, W. L. Cody, J. X. He, C. Humblet, G. H. Lu, E. Lunney, T. C. Major, R. L. Panek, P. Schelkun, R. Skeean and G. R. Marshall, J. Med. Chem., 36, 1902 (1993).
59. G. V. Nikiforovich, J. L.-F. Kao, K. Plucinska, W. J. Zhang and G. R. Marshall, Biochemistry, 33, 3591 (1994).
60. S. C. Lee, A. F. Russel and W. D. Laidig, Int. J. Pept. Prot. Res., 35, 367 (1990).
61. D. J. Kyle, P. R. Blake, D. Smithwick, J. A. Martin, J. A. Sinsko and M. F. Summers, J. Med. Chem., 36, 1450 (1993).
62. A. Otter, P. Bigler, J. M. Stewart and G. Kotovych, Biopolymers, 33, 769 (1993).
63. R. J. Vavrek and J. M. Stewart, Peptides, 6, 161 (1985).
64. D. J. Kyle, L. M. Green, P. R. Blake, D. Smithwick and M. F. Summers, Peptide Res., 5, 206 (1992).
65. F. J. Hock, K. Wirth, U. Albus, W. Linz, H. J. Gerhards, G. Wiemer, St. Henke, G. Breipohl, W. Konig, J. Knolle and B. A. Scholkens, Br. J. Pharmacol., 102, 769 (1991).
66. S. Chakravarty, D. Wilkins and D. J. Kyle, J. Med. Chem., 36, 2569 (1993).
67. S. Chakravarty, M. Connolly and D. J. Kyle, Peptide Res., 8, 16 (1995).
68. J. L. Krstenansky, T. Ho, R. Tahilramani, J. H. B. Pease, S. Bhakta, H. Ostrelich and K. Jarnagin, Lett. Peptide Sci., 2, 000 (1995).
69. K. Jarnagin, S. Bhakta, J. Pease and R. Freedman, FASEB J., 8, A352 (1994).
70. G. M. Smith and D. F. Veber, Biochem. Biophys. Res. Comm., 134, 907 (1986).
71. R. A. Dammkoehler, S. F. Karasek, E. F. B. Shands and G. R. Marshall, J. Comput.-Aided Mol. Design, 3, 3 (1989).
72. G. M. Crippen and T. F. Havel, "Distance Geometry and Molecular Conformation," Research Studies Press, New York, 1988.
73. C. E. Peishoff, J. S. Dixon and K. D. Kopple, Biopolymers, 30, 45 (1990).
74. T. Noguti and N. Go, Biopolymers, 24, 527 (1985).
75. G. Chang, W. C. Guida and W. C. Still, J. Am. Chem. Soc., 111, 4379 (1989).
76. R. D. Cramer III, D. E. Patterson and J. D. Bunce, J. Am. Chem. Soc., 110, 5959 (1988).

77. R. P. Sheridan, R. Nilakantan, J. S. Dixon and R. Venkataraghavan, J. Med. Chem., 29, 899 (1986).
78. R. S. McDowell, T. R. Gadek, P. L. Barker, D. J. Burdick, K. S. Chan, C. L. Quan, M. Struble, E. D. Thorsett, M. Tischler, J. Y. K. Tom, T. R. Webb and J. P. Burnier, J. Am. Chem. Soc., 116, 5069 (1994).
79. C. Y. Bowers, F. A. Momany, G. A. Reynolds and A. Hong, Endocrinology, 114, 1537 (1984).
80. F. A. Momany, C. Y. Bowers, G. A. Reynolds, D. Chang, A. Hong and K. Newlander, Endocrinology, 108, 31 (1981).
81. F. A. Momany, C. Y. Bowers, G. A. Reynolds, A. Hong and K. Newlander, Endocrinology, 114, 1531 (1984).
82. R. S. McDowell, K. A. Elias, M. S. Stanley, D. J. Burdick, J. P. Burnier, K. S. Chan, W. J. Fairbrother, R. G. Hammonds, G. S. Ingle, N. Jacobsen, D. L. Mortensen, T. E. Rawson, W. B. Won, R. G. Clark and T. C. Somers, submitted.
83. P. J. Casey, P. A. Solski, C. J. Der and J. E. Buss, Proc. Natl. Acad. Sci. USA, 86, 8323 (1989).
84. J. F. Hancock, A. I. Magee, J. E. Childs and C. J. Marshall, Cell, 57, 1167 (1989).
85. W. R. Schafer, R. Kim, R. Sterne, J. Thorner, S. H. Kim and J. Rine, Science, 245, 379 (1989).
86. Y. Reiss, S. J. Stradley, L. M. Gierasch, M. S. Brown and J. L. Goldstein, Proc. Natl. Acad. Sci. U.S.A., 88, 732 (1991).
87. J. C. Marsters Jr., R. S. McDowell, M. E. Reynolds, D. A. Oare, T. C. Somers, M. S. Stanley, T. E. Rawson, M. E. Struble, D. J. Burdick, K. S. Chan, C. M. Duarte, K. J. Paris, J. Y. K. Tom, D. T. Wan, Y. Xue and J. P. Burnier, Bioorg. Med. Chem., 2, 949 (1994).
88. G. L. James, J. L. Goldstein, M. S. Brown, T. E. Rawson, T. C. Somers, R. S. McDowell, C. W. Crowley, B. K. Lucas, A. D. Levinson and J. C. Marsters Jr, Science, 260, 1937 (1993).
89. W. R. Kester and B. W. Matthews, Biochemistry, 16, 2506 (1977).

Chapter 29. Applications of Biosensor Technology in Drug Discovery

David J. Matthews
Arris Pharmaceutical Corporation
South San Francisco, CA 94080

Introduction - Characterization of intermolecular interactions is an essential component of many modern drug discovery programs. Virtually all biological processes which are the target of pharmaceutical intervention involve interactions between two or more biomolecules, for example, binding of a growth factor to its cellular receptor, an antibody to its antigen or a protease to its substrate or an inhibitor. In the case of biopharmaceuticals, one of the macromolecules may itself be used as a therapeutic agent (for example, growth factors or antibodies); alternatively, it may be possible to discover a small molecule which acts either as an agonist or antagonist. In both cases, it is essential to obtain detailed information on the nature of the interaction. This may involve study of the cellular response to a given compound and/or quantitation of the binding affinities involved. Recently, biosensor techniques which measure physical changes at the molecular or cellular level have emerged as powerful new tools for studying biospecific interactions. The commercialization of optical biosensors has provided researchers with versatile devices for direct measurement of intermolecular interactions. Using these instruments, it is possible to qualitatively characterize binding interactions in real time: in addition, one may rapidly obtain quantitative information about an interaction (for example, on- and off-rates and affinity constants). Furthermore, the recent introduction of a highly sensitive potentiometric pH sensor (dubbed the microphysiometer or cytosensor) allows one to monitor minute changes in pH induced by cellular metabolism. These measurements can provide valuable information on the physiological responses of many different cell types to an even more diverse range of receptor specific agonists and antagonists. This review will concentrate on aspects of the optical biosensor and the microphysiometer which are of particular relevance to the drug discovery process.

Principles of Biosensors - Most biosensor devices consist of (i) a surface to which molecules are attached, resulting in a biological sensing element and (ii) a signal transduction system, which can detect changes when a sample is added and produce a measurable signal (1-5). Surface chemistry is thus an important factor in biosensor systems, since the method of coupling a biospecific molecule to a surface must be closely linked to the signal transduction process (6). The biological sensing component of a biosensor system may consist of an enzyme, an antibody or receptor protein or whole cells (7). Enzyme based systems have proved to be highly effective for detection of specific analytes, but are of limited use in drug discovery applications due to their inherent specificity for a single chemical reaction. The detection systems for enzyme-based biosensors are usually dependent on the catalytic generation of a detectable product (for example, a redox-active or optically active product) and must, in general, be individually designed for specific applications. Antibody and receptor-based systems have potentially broader application, although their success is critically dependent on implementation of a generally applicable method of signal detection and transduction. Whole cell biosensors are attractive in that assays employing whole cells demonstrate the response of a living cell to a particular compound, rather than an isolated receptor molecule (8,9). However, animal cells (which are of the most interest for pharmaceutical applications) are relatively fragile and may exhibit a wide variety of physiological responses to external stimuli. In this case too, the method of signal detection and transduction is critical. Most signal transduction systems in current use are electrically or optically based. (Calorimetric sensors (10) and acoustic (piezoelectric) devices (11,12) have also been investigated, but have not yet been developed to a stage where they can be used on a

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

routine basis). Electrochemical biosensors transduce an electrical signal when a biological interaction occurs, usually either in the form of a current (amperometric) or a potential difference (potentiometric). The first such biosensors used immobilized glucose oxidase to measure glucose in solution (1,13): many devices have since been developed which rely on a redox reaction and amperometric or potentiometric detection (14-20). Usually, these electrochemical devices are coupled to enzymatic processes and thus are highly specialized for detection of one particular analyte. However, the microphysiometer, or cytosensor, is a biosensor device which does not rely on surface interactions to produce a signal. It is a form of potentiometric biosensor which measures minute pH changes caused by cells in a sample chamber and may be applied to the study of many biological processes (21). Perhaps the most promising form of signal transduction currently available for studying intermolecular interactions is offered by the optical biosensor, which has been developed commercially by several manufacturers (22). Measurable optical properties include light scattering or absorption: however, the most widely used technique involves changes in refractive index. Applications of optical biosensors are described in detail below.

OPTICAL BIOSENSORS

Principles of Optical Biosensors - Currently, the most widely used signal transduction technology for general application to the study of intermolecular interactions and drug discovery is optically based signal transduction (22,23). Optical biosensor systems rely on the behavior of light at a boundary between media of different refractive index. When light is incident on an interface of higher to lower refractive index (such as that between a sensor and a biological solution), the light will be totally reflected if it is incident beyond a certain critical angle. When total internal reflection occurs, an evanescent wave will be propagated into the lower refractive index medium, decaying exponentially within a few hundred nanometers. It is possible to probe this evanescent wave via resonance phenomena, and one of the major differences between commercially available optical biosensor systems is the method used to detect changes in the evanescent field (22,23). For example, if a thin gold film is inserted at the interface between the sensor and the solution, free electrons in the gold surface can resonate with and absorb energy from incident light at a specific angle (termed surface plasmon resonance (24-26)). The energy absorption at the surface is manifest as a decrease in the reflected light intensity (Fig. 1).

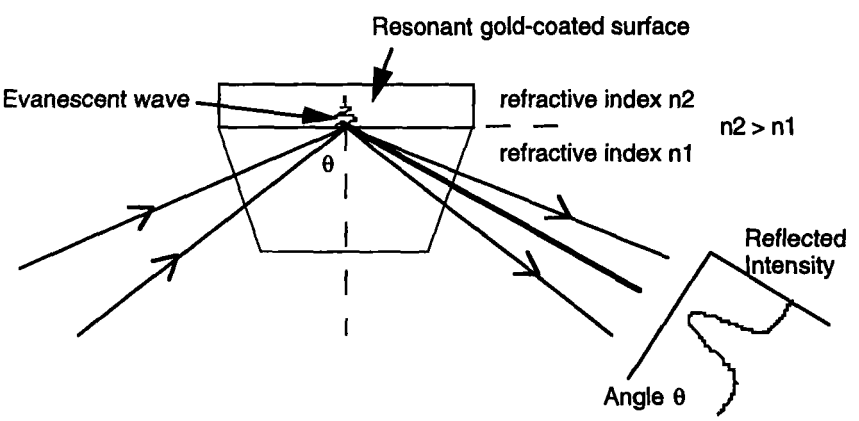


Fig. 1 Schematic diagram of a surface plasmon resonance optical biosensor

Alternatively, the incident light may be coupled into a waveguide, wherein at certain angles of incidence it undergoes multiple total internal reflection (27,28). On reflection, the light undergoes a 2π phase change. Since the transverse electric and magnetic components of the light undergo this phase change at different angles, the effect can be measured using interference between the two components. For both surface plasmon resonance and waveguide techniques, the precise angle of resonance is critically dependent on the thickness and refractive index of the boundary. Hence, binding of molecules near to the interface results in a change in the resonance angle which is proportional to the number and mass of molecules bound.

Surface Chemistries for Ligand Immobilization - In commercially available optical biosensors, a carboxymethylated dextran layer is often coupled to the sensor surface (29,30). This allows immobilization of molecules by a variety of coupling chemistries and provides a hydrophilic environment which promotes biological binding while reducing nonspecific hydrophobic interactions (31,32). The most widely used immobilization technique is amine coupling. The carboxymethylated dextran surface is derivatized using N-hydroxysuccinimide (NHS), mediated by N-ethyl-N'-(dimethylaminopropyl) carbodiimide (EDC) (33). The NHS esters can subsequently react with primary amino groups on target proteins. As an extension of this technique, streptavidin may be immobilized via amine coupling and biotinylated molecules subsequently coupled to the streptavidin surface. A more specific means of coupling is afforded by thiol coupling, allowing immobilization of ligands via free cysteine residues. In this method, the biosensor surface is also derivatized with EDC/NHS, then active disulfides are coupled to the surface (for example, using the thiol specific coupling agent 2-(2-pyridinyldithio)ethaneamine (PDEA)). Proteins containing free thiols (either naturally occurring or introduced via mutagenesis (34,35) or by using Traut's reagent (36)) may then be coupled to the surface via a disulfide exchange reaction. A third option for ligand immobilization employs aldehyde coupling to activated hydrazide surfaces. This technique requires introduction of aldehyde groups into the ligand, for example, by periodate oxidation of cis-diols present in polysaccharide side chains (37,38). Hydrazine is coupled to an EDC/NHS activated surface and reacted with aldehydes on the target protein. Recently, another immobilization chemistry has been described which does not rely on the use of a carboxymethylated dextran layer (28,39). Instead, the sensor surface is directly derivatized with functional groups to which proteins may be directly adsorbed. Such surfaces are useful for cell binding studies, since steric hindrance in carboxymethylated dextran-coated surfaces may impede binding of whole cells (39).

Data Collection and Analysis - Fundamentally, binding interactions at the biosensor surface occur under one of two regimes: mass transport limited binding or kinetic limited binding (40,41). Mass transport limited binding occurs when very high densities of ligand are immobilized on the surface, such that pseudo-first order binding kinetics are observed. In this case, the rate of binding to the surface is limited only by diffusion/mass transport phenomena and is proportional to the concentration of analyte in solution. This form of interaction is particularly useful for quantitation assays where high sensitivity is required. For measurement of kinetic parameters, it is necessary to immobilize relatively low amounts of ligand on the biosensor surface. In this case it is possible to measure association rate constants, provided they are lower than the mass transport limit. Generally, for macromolecular interactions, association constants from 10^2 - $5x10^6$ $M^{-1}s^{-1}$ and dissociation constants from 10^{-2} to 10^{-5} s^{-1} may be effectively measured (41). Following injection and dissociation of analytes, any residual analyte may be eluted from the biosensor surface using an appropriate regeneration solution. Depending on the immobilized ligand and the regeneration conditions used, it may be possible to re-use the same sensor surface for at least 100 measurements (42). Data for 1:1 interaction kinetics are usually analyzed by fitting to a simple Langmuir isotherm. Several techniques exist for exploring more complex binding models involving competition or cooperative effects, including global fitting methods (43,44). The use of various mathematical models to interpret binding data has

been discussed at length, as have the pitfalls associated with mass transport limitations, linear transformation of data and incorrect assumptions in the binding model (40,41,43,45,46). As well as direct measurement of binding kinetics, relative affinities may be investigated using competition assays. Such assays are particularly useful for small molecules which are not easily detected by direct binding. This technique has been successfully applied to families of peptides which bind to SH2 domains (47-49) and binding of peptides to major histocompatibility complex 1 (MHC-1) molecules (50).

Applications of Optical Biosensors - Since the inception of commercially available optical biosensors, the most extensive use of these instruments has been made in the field of protein-protein interactions. Historically, many different techniques have been used to quantify intermolecular interactions, for example, displacement of radiolabeled ligands bound to cells or purified receptors. Most of these methods involve measurement of a complex formed between the binding components at equilibrium, and thus do not consider the dynamic process of ligand binding and dissociation. Optical biosensor techniques allow one to measure the kinetics of association and dissociation between macromolecules in addition to steady state binding equilibria, thus providing a rich source of information concerning the interaction. Specific applications of optical biosensor measurements are discussed below.

Antibodies - Optical biosensor techniques are well suited to the measurement of antibody-antigen interactions. The resonance signal obtained is proportional to the mass of the binding ligand, so large analytes such as immunoglobulins give relatively high signals. Surface plasmon resonance has been successfully used as an alternative to ELISA, radioimmunoassay and slot blot methods for characterizing kinetics of antibody-antigen interactions (51-54). The interactions of anti-peptide antibodies with both peptides and the cognate protein have also been compared, demonstrating that in some cases the equilibrium constant for peptide binding is substantially lower than that for binding the cognate protein (55-57). Another useful application is epitope mapping (58-60). In this technique, one may immobilize an antigen on the biosensor surface, make successive injections of different antibodies and determine whether any of the antibodies compete for the same antigen binding site. Surface plasmon resonance has also been used to functionally characterize antibodies of potential therapeutic use, for example, a humanized version of the anti-p185HER2 antibody 4D5 (61).

Receptor-Ligand Interactions - Surface plasmon resonance has been used to provide detailed information about the interaction of polypeptide hormones with their receptors. The 1:2 stoichiometry of the human growth hormone (hGH):hGH receptor interaction was confirmed by biosensor analysis, using 2 mutants of the extracellular domain of the hGH receptor which either allowed or prevented receptor dimerization (34). Furthermore, by a combination of site-directed mutagenesis and biosensor analysis, the roles in the binding interaction of specific hGH and hGH receptor amino acid residues were determined (34,62). Most of the effects observed were due to changes in the off-rates of mutant proteins, although several mutants showed a decrease in on-rate. Surface plasmon resonance studies with epidermal growth factor (EGF) and the extracellular domain of its receptor have also provided evidence for receptor dimerization (63). Biosensor measurements showed that the off-rate for the interaction of EGF with cross-linked dimeric receptor was about 10 times slower than for the interaction with monomeric receptor. The interleukin IL-6/IL-6 receptor system has been extensively studied, demonstrating ternary complex formation between IL-6, IL-6 receptor and the signal transduction protein gp130 (64-66). Surface plasmon resonance techniques were also used to characterize the relatively weak interactions between cell adhesion molecules (CAMs). K_D values of 60-90 μM were determined for the rat CAMs CD2 and CD48, and the low affinity was shown to be due to an extremely high off-rate (67). Some other systems successfully probed using optical biosensor

techniques include interleukin IL-5/IL-5 receptor (68) and fibroblast growth factor/heparin (69).

Cellular Signal Transduction - Surface plasmon resonance has been used to probe the interactions of Src-homology 2 (SH2) domains with phosphorylated peptides. A variety of SH2 domains have been expressed as fusion proteins in *E. coli* and assayed for direct binding to peptides immobilized on the biosensor surface (47-49, 70-72). These include the SH2 domains from Src and Lck tyrosine kinases (48), the p85 subunit of phosphatidylinositol 3-kinase (47,49,70,71), Grb-2 (71) and phospholipase Cγ (71). In one of these cases, the biosensor experiments were compared to ^{125}I phosphopeptide displacement assays and showed good correlation (48). Competition assays have also been used to determine IC_{50} values for peptides in several different systems, allowing rapid comparison of many different peptides (47-49). Furthermore, kinetic parameters for SH2 domain-phosphopeptide binding have been obtained (47,48,70,71).The measurements indicate high on-rates coupled with very fast off-rates, and also demonstrate the high specificity of SH2 domain-phosphopeptide interactions. Many of the above studies emphasize the importance of testing the effect of competing ligand on the dissociation rate: since both the on-rate and off-rates are fast, rebinding phenomena can dramatically affect the off-rate estimation in the absence of competing ligand.

Identification of Novel Ligands - The use of an optical biosensor system to isolate novel ligands for orphan receptors has also been reported (73). The extracellular domain of the ECK receptor protein tyrosine kinase was immobilized on a biosensor surface and concentrated cell culture supernatants from a variety of cell lines were injected onto the surface. A binding response was measured from several specific supernatants, allowing rapid identification of samples containing the ligand. Biosensor analysis has also been used to characterize peptides displayed on phage particles which were isolated from filamentous phage display libraries (74).

THE MICROPHYSIOMETER

Operational Principles - The microphysiometer, or cytosensor, detects perturbations in the metabolic activity of living cells by monitoring the rate at which cells excrete metabolic acid byproducts (21). It is thus a pH sensor, and uses a light-addressable potentiometric sensor (LAPS) to detect changes in extracellular pH induced by cells within a sample chamber (75). Such a technique is very general in scope, and with appropriate control experiments it is feasible to monitor the effect of a compound on specific cellular receptors. The device is outlined schematically in Fig. 2. The sample chamber is connected to a doped silicon layer via a thin insulator, and an external circuit controls the potential from the silicon layer to the aqueous sample. When the silicon is illuminated with a light emitting diode, an alternating photocurrent is generated in the external circuit. The magnitude of this current depends on the potential at the interface between the aqueous sample and the insulating layer required to reverse bias the silicon layer. This potential in turn depends on the electrostatic potential due to charged molecules or ions bound to the insulating layer. Since the insulating layer can bind protons, the current in the external circuit is pH dependent. The system can thus be used to measure any process which results in pH changes within the sample chamber, for example, cellular metabolism. In particular, ATP synthesis via a number of metabolic pathways results in proton production and protons are excreted from cells via various proton channels and ion pumps. Receptor activation may alter the rate of extracellular acidification by modulating cellular metabolism and/or ion transport. Experiments are performed by perfusing a flow chamber containing cells with culture medium containing the ligand of interest. Due to the fact that the response depends on pH changes in the extracellular medium, experiments are usually performed in media with relatively low buffering capacity, about 1mM. It has also been shown that pre-starvation of cells in serum free medium

may increase the sensitivity of the response (76). Since the medium is continually being exchanged, the pH of the flow chamber remains relatively constant during perfusion: however, when perfusion is stopped the pH of the medium decreases. The rate of acidification is measured by the biosensor and can be used to generate dose-response data. Typically, activation of a cellular receptor is observed in the microphysiometer as a transient peak in the extracellular acidification rate followed by a new steady state level of acidification rate. One possible interpretation of the transient effects is a lag in the rate of ATP synthesis relative to the rate of ATP hydrolysis as the overall ATP flux increases in response to receptor activation. Another source of transient effects may involve changes in the activity of proton pumps (21). The operation and applications of the microphysiometer have been discussed in more detail elsewhere (75-80).

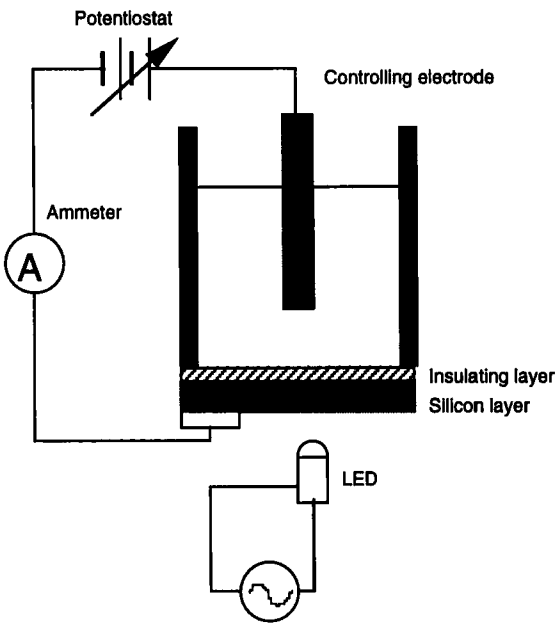


Fig. 2 Schematic diagram of the cytosensor/microphysiometer

Study of Cellular Responses - The major attraction of the microphysiometer is that it is allows measurement of the responses of many different cell types to a wide variety of receptor ligands. The cellular receptors may in turn be coupled to many diverse intracellular signaling pathways. Due to the wide variety of cellular responses elicited by diverse ligand-receptor and second messenger systems, the magnitude and kinetics of responses may vary significantly (76-78,80). Nevertheless, microphysiometer measurements have been shown to yield similar results to a wide variety of conventional assays, including cell proliferation, neurite outgrowth, ion current and second messenger quantitation assays (81,82). Examples of cytokines and growth factors studied using this technique include insulin (80), epidermal growth factor (76,77), IL-2 (83-84) and GM-CSF (78,85,86), although perhaps the most extensively studied receptor superfamily to date is the G-protein coupled receptor family, whose members include cholinergic (76,78,80,87), dopaminergic (88-90) and adrenergic receptors (80,91). Other systems analyzed include neurotrophic factors and their receptors (92) and T-cell activators (84,93). Another intriguing application is the study of viral cytopathology. Studies with vesicular stomatitis virus (VSV) and human immunodeficiency virus-1 (HIV-1) have shown that viral infection can lead to a

decrease in cell proliferation resulting in a decrease in acidification detected by the cytosensor (77,78). In some cases, microphysiometer assays may also serve as an alternative to animal testing in evaluation of irritancy and toxicity of compounds (77, 94-96). Such studies involve measuring the inhibition of extracellular acidification by irritants, and significant correlation has been observed between results from microphysiometer assays and *in vivo* effects (76). Metabolic effects are observed on a time scale of minutes and it is possible to measure not only immediate depression of metabolic rates but the ability of cells to recover from exposure to irritants. A related application is the measurement of the effects of cytotoxic drugs (for example, chemotherapeutics) on different cell lines (76).

Conclusions and Future Directions - Several aspects of biosensor technology have recently emerged as a powerful new tools for understanding biological problems which are central to drug discovery efforts. This review has focused on optical biosensors and the microphysiometer, two biosensor technologies which have recently become commercially available and thus accessible to researchers in the field of drug discovery and design. These methods offer novel analytical approaches to a wide range of problems as described above. In the future, the growth of biosensor technology as a tool for drug discovery and characterization of biomolecular interactions is likely to be driven by improvements in instrumentation and peripheral technologies. For example, in the field of cellular biosensors, recent work has demonstrated the feasibility of combining microcapillary chromatography with physiological interrogation of single cells to identify bioactive ligands in minute volumes of complex solutions (97). Optical biosensors (in the form of evanescent wave probes) are now an established tool for studying molecular interactions, and a second-generation surface plasmon resonance detector ("BIAcore 2000") has recently been introduced (98). This offers greatly enhanced sensitivity and signal-to-noise ratio compared to previous instruments and allows simultaneous interaction analysis on four different biosensor surfaces. It has already been shown that this instrument can detect very small compounds (for example, binding of theophylline (MW 180 Da) to an anti-theophylline antibody (98)). This greatly broadens the scope of optical biosensor technology and may enable the technique to be used for high-throughput screening of chemical libraries.

References

1. A.P.F. Turner, I. Karube and G.S. Wilson, "Biosensors: Fundamentals and Applications", Oxford University Press, Oxford, England, 1987
2. A.E.G. Cass, "Biosensors: A Practical Approach", IRL Press, Oxford, England, 1990
3. E.A.H. Hall, "Biosensors", Open University Press, Buckingham, England, 1990
4. D. Griffiths and G. Hall, Trends in Biotechnol. 11, 122-130 (1993)
5. J.N. Roe, Pharm. Res. 9, 835 (1992)
6. R. F. Taylor in "Protein Immobilization: Fundamentals and Applications", Marcel Dekker, NY., 1991, p. 263
7. M.P. Byfield and R.A. Abuknesha, Biosens. Bioelectron. 9, 373 (1994)
8. C.A. Corcoran and G.A. Rechnitz, Trends in Biotechnol. 3, 92 (1985)
9. D.C. Wijesuriya and G.A. Rechnitz, Biosens. Bioelectron. 8, 155 (1993)
10. M.J. Muehlbauer, E.J. Guilbeau and B.C. Towe, Anal. Chem. 61, 77 (1989)
11. E. Prusak-Sochaczewski, J. Luong and G. Guilbault, Enzyme Microb. Tech. 12, 173 (1990)
12. E. Prusak-Sochaczewski and J. Luong , Anal. Lett. 23, 183 (1990)
13. L.C. Clark and C. Lyons, Ann. N.Y. Acad. Sci. 148, 133 (1962)
14. A.E.G. Cass, G. Davis, G.D. Francis, H.A..O. Hill, I.J. Higgins, E.V. Plotkin, L.D.L. Scott and A.P.F. Turner, Anal. Chem. 56, 667 (1984)
15. H. Suzuki, E. Tamiya and I. Karube, Anal. Chim. Acta. 199, 85 (1987)
16. B.A. Gregg and A. Heller, Anal. Chem. 62, 258 (1990)
17. N. Sugao, M. Sugawara, H. Minami, M. Uto and Y. Umezawa, Anal. Chem. 65, 363 (1993)
18. I. Karube and S. Suzuki, Ion-selective Electrode Rev. 6, 15 (1984)
19. T. Yao and G.A. Rechnitz, Anal. Chem. 59, 2115 (1987)
20. G.F. Blackburn, D.B. Talley, P.M. Booth, C.N. Durfor, M.T. Martin, A.D. Napper and A.R. Rees, Anal. Chem. 59, 2115 (1990)

21. J. Owicki and J. Parce, Biosens.Bioelectron. 7, 255 (1992)
22. J. Hodgson, Biotechnology 12, 31 (1994)
23. G. Panayotou, M.D. Waterfield and P. End, Curr. Biol. 3, 913 (1993)
24. C.S. Mayo and R.B. Hallock, J. Immunol. Methods 120, 105 (1989)
25. L. Fägerstam in "Techniques in Protein Chemistry II", ed. J.J. Villafranca, Academic Press, New York, N.Y., 1991, p. 65
26. U. Jönsson, L. Fägerstam, B. Ivarsson, K. Lundh, S. Löfås, B. Persson, H. Roos, I. Rönnberg, S. Sjölander, E. Stenberg, R. Ståhlberg, C. Urbaniczky, H. Östlin and M. Malmqvist, Biotechniques 11, 620 (1991)
27. R. Cush, J.M. Cronin, W.J. Stewart, C.H. Maule, J. Molloy and N.J. Goddard, Biosens. Bioelectron. 8, 347 (1993)
28.. P.E. Buckle, R.J. Davies, T. Kinning, D. Yeung, P.R. Edwards, D. Pollard-Knight and C.R. Lowe, Biosens. Bioelectron. 8, 355 (1993)
29. S. Löfås and B. Johnsson, J. Chem. Soc. Chem. Commun. 21, 1526 (1990)
30. E. Stenberg, B. Persson, H. Roos and C. Urbaniczky, J. of Colloid. Interface Science 143, 513 (1991)
31. B. Johnsson, S. Löfås and G. Lindquist, Anal. Biochem. 198, 268 (1991)
32. D.J.O'Shannessy, M. Brigham-Burke and K. Peck, Anal. Biochem. 205, 132 (1992)
33. P. Cuatrecasas and I. Parikh, Biochemistry 11, 2291 (1972)
34. B.C. Cunningham and J.A. Wells, J. Mol. Biol. 234, 554 (1993)
35. R.F. Kelley, Methods:Companion Meth. Enzymol. 6, 111 (1994)
36. J.M. Lambert, R. Jue and R.R. Traut, Biochemistry 17, 5406 (1978)
37. L. Van Lenten and G. Ashwell, J. Biol. Chem. 246, 1889 (1971)
38. D.J. O'Shannessy and M. Wilcheck, Anal. Biochem. 191, 1 (1990)
39. H.J. Watts, C.R. Lowe and D.V. Pollard-Knight, Anal. Chem. 66, 2465 (1994)
40. R.W. Glaser, Anal. Biochem. 213, 152 (1993)
41. R. Karlsson, H. Roos, L. Fägerstam and B. Persson, Methods: Companion Meth. Enzymol. 6, 99 (1994)
42. M. Brigham-Burke, J.R. Edwards and D.J. O'Shannessy, Anal. Biochem. 205, 125 (1992)
43. R.J. Fisher and M. Fivash, Curr. Opin. Biotech. 5, 389 (1994)
44. R.J. Fisher, M. Fivash, J. Casas-Finet, J.W. Erickson, A. Kondoh, S.V. Bladen, C. Fisher, D. Watson and T. Papas, Protein Sci. 3, 257 (1994)
45. D.J. O'Shannessy, B.M. Brigham, K.K. Soneson, P. Hensley and I. Brooks, Anal. Biochem. 212, 457 (1993)
46. D. O'Shannessy , Curr. Opin. Biotech. 5, 65 (1994)
47. S. Felder , M. Zhou, P. Hu, J. Ureña, A. Ullrich, M. Chaudhuri, M. White, S.E. Shoelson and J. Schlessinger, Mol. Cell. Biol. 13, 1449 (1993)
48. G. Payne, S.E. Shoelson, G.D. Gish, T. Pawson and C.T. Walsh, Proc. Natl. Acad. Sci. USA 90, 4902 (1993)
49. C. Ponzetto, A. Bardelli, F. Maina, P. Longati, G. Panayotou, R. Dhand, M.D. Waterfield and P.M. Comoglio, Mol. Cell. Biol. 13, 4600 (1993)
50. S.N. Khilko, M. Corr, L.F. Boyd, A. Lees, J.K. Inman and D.H. Margulies, J. Biol. Chem. 268, 15425 (1993)
51. R. Karlsson, A. Michaelsson and L. Mattsson, J. Immunol. Methods 145, 229 (1991)
52. A. -C. Malmborg, A. Michaëlsson, M. Ohlin, B. Jansson and C.A.K. Borrebaeck, Scand. J. Immunol. 35, 643 (1992)
53. M. Malmqvist, Curr. Opin. Immunol. 5, 282 (1993)
54. L. C. Gruen, J.L. McKimm-Breschkin, J.B. Caldwell and E.C. Nice, J. Immunol. Methods 168, 91 (1994)
55. D. Altschuh, M.C. Dubs, E. Weiss, G. Zeder-Lutz and M.H.V. Van Regenmortel, Biochemistry 31, 6298 (1992)
56. M.C. Dubs, D. Altschuh and M.H.V. Van Regenmortel, J. Chromat. 597, 127 (1992)
57. G. Zeder-Lutz, D. Altschuh, H.M. Geysen, E. Trifilieff, G. Sommermeyer, M.H.V. Van Regenmortel, Mol. Immunol. 30, 145 (1993)
58. L.G. Fägerstam, Å. Frostell, R. Karlsson, M. Kullman, A. Larsson, M. Malmqvist and H. Butt, J. Mol. Recogn. 3, 208 (1990)
59. E. Nice, J. Layton, L. Fabri, U. Hellman, A. Engstrom, B. Persson and A.W. Burgess, J. Chromatography 646, 159 (1993)
60. J.L. Daiss and E.R. Scalice, Methods: Companion Meth. Enzymol. 6, 143 (1994)
61. R. Kelley and M. O'Connell, M. Biochemistry 32, 6828 (1993)
62. T. Clackson and J. Wells, Science 267, 383 (1995)
63. M. Zhou, S. Felder, M. Rubinstein, D.R. Hurwitz, A. Ullrich, I. Lax and J. Schlessinger. Biochemistry 32, 8193 (1993)
64. K. Yasukawa, K. Futatsugi, T. Saito, H. Yawata, M. Narazaki, H. Suzuki, T. Tage and T. Kishimoto, Immunol. Lett. 31, 123 (1992)

65. L.D. Ward, A. Hammacher, J. Chang, J-G. Zhang, G. Discolo, R.L. Moritz, K. Yasukawa and R.J. Simpson in "Techniques in Protein Chemistry V", ed. J.W. Crabb, Academic Press, New York, N.Y., 1994 p. 331
66. L.D. Ward, A. Hammacher, J-G. Zhang, J. Weinstock, K. Yasukawa, C.J. Morton, R.S. Norton and R.J. Simpson, Protein Sci. 2, 1472 (1993)
67. P.A. van der Merwe, M.H. Brown, S.J. Davis and A.N. Barclay, EMBO J. 12, 4945 (1993)
68. T.A. Morton, D.B. Bennett, E.R. Applebaum, D.M. Cusimano, K.O. Johanson, R.E. Matico, P.R. Young, M. Doyle and I.M. Chaiken, J. Mol. Recogn. 7, 47 (1994)
69. H. Mach, D.B. Volkin, C.J. Burke, C.R. Midaugh, R.J. Linhardt, J.R. Fromm, D. Loganathan and L. Mattson, Biochemistry 32, 5480 (1993)
70. G. Panayotou, G. Gish, P. End, O. Truong, I. Gout, R. Dhand, M.J. Fry, I. Hiles, T. Pawson and M.D. Waterfield, Mol. Cell. Biol. 13, 3567 (1993)
71. C. Ponzetto, A. Bardelli, Z. Zhen, F. Maina, P. dalla Zonca, S. Giordano, A. Graziani, G. Panayotou and P.M. Comoglio, Cell 77, 261 (1994)
72. L.E.M. Marengere, Z. Songyang, G.D. Gish, M.D. Schaller, J.T. Parsons, M.J. Stern, L.C. Cantley and T. Pawson, Nature 369, 502 (1994)
73. T.D. Bartley , R.W. Hunt, A.A. Welcher, W.J. Boyle, V.P.Parker, R.A. Lindberg, H.S. Lu, A.M. Colombero, R.L. Elliott, B.A. Guthrie, P.L. Holst, J.D. Skrine, R.J. Toso, M. Zhang, E. Fernandez, G. Trail, B. Varnum, Y. Yarden, T. Hunter and G.M. Fox, Nature 368, 558 (1994)
74. E. Lasonder, G.A. Schellekens and G.W. Welling, Nuc. Ac. Res. 22, 545 (1994)
75. D.G. Hafeman, J.W. Parce and H.M.McConnell (1988) Science 240, 1182
76. J.C. Owicki, J.W. Parce, K.M. Kercso, G.B. Sigal, V.C. Muir, J.C. Venter, C.M. Fraser and H.M. McConnell, Proc. Natl. Acad. Sci. 87, 4007 (1990)
77. J.W. Parce, J.C. Owicki, K.M. Kercso, G.B. Sigal, H.G. Wada, V.C. Muir, L.J. Bousse, K.L. Ross, B.I. Sikic and H.M. McConnell, Science 246, 243 (1989)
78. H.M. McConnell J.C. Owicki, J.W. Parce, D.L. Miller, G.T. Baxter, H.G. Wada and S. Pitchford, Science 257, 1906 (1992)
79. M. Sartore, M. Adami and C. Nicolini, Biosens. Bioelectron. 7, 57 (1992)
80. H.M. McConnell, P. Rice, G.H. Wada, J.C. Owicki and J.W. Parce, Curr.Opin.Struct.Biol. 1, 647 (1991)
81. M. Hirst and S. Pitchford, J. NIH. Res. 5, 69 (1994)
82. S. Pitchford and M. Hirst, J. NIH. Res. 6, 84 (1994)
83. H.G. Wada, J.C. Owicki and J.W. Parce, Clin. Chem. 37, 600 (1991)
84. B. Nag, H.G. Wada, K.S. Fok, D.J. Green, S.D. Sharma, B.R. Clark, J.W. PArce and H.M. McConnell, J. Immunol. 148, 2040 (1992)
85. G.T. Baxter, D.L. Miller, R.C. Kuo, H.G. Wada and J.C. Owicki, Biochemistry 31, 10950 (1992)
86. H.G. Wada, R. Indelicato, L. Meyer, T. Kitamura, A. Miyajima, G. Kirk, V.C. Muir and J.W. Parce, J. Cell. Physiol. 154, 129 (1993)
87. D.L. Miller, J.C. Olson, J.W. Parce and J.C. Owicki, Biophys J. 64, 813 (1993)
88. M.P. Rosser, M.R. Kozlowski and K.A. Neve, Soc. Neurosci. Abstr. 17, 818 (1991)
89. K.A. Neve, M.R. Kozlowski and M.P. Rosser, J. Biol. Chem. 267, 25748 (1992)
90. C.L. Chio, M.E. Lajiness and R.M. Huff, Mol. Pharmacol. 45, 51 (1993)
91. S. Skwish and K.E.J. Dickinson, FASEB J. 8, A650 (1994)
92. R.M. Johnson, P.A. McNeely, K. De Moor, G.R. Stewart, B.S. Glaeser and S. Pitchford, Brain Res. 646, 327 (1994)
93. B. Nag, H.G. Wada, S.V. Deshpande, D. Passmore, T. Kendrick, S.D. Sharma, B.R. Clark and H.M. McConnell, Proc. Natl. Acad. Sci. USA 90, 1604 (1993)
94. L.H. Bruner, K.R. Miller, J.C. Owicki, J.W. Parce and V.C. Muir, Toxic. In Vitro 5, 277 (1991)
95. D.M. Bagley, L.H. Bruner, O. DeSilva, M. Cottin, K.A.F. O'Brien, M. Uttley and A.P. Walker, Toxic. In Vitro 6, 275 (1992)
96. P. Catroux, A. Rougier, K.G. Dossou and M. Cottin, Toxic. In Vitro 7, 465 (1993)
97. J.B. Shear, H.A. Fishman, N.L. Allbritton, D. Garigan, R.N. Zare and R.H. Scheller, Science 267, 74 (1995)
98. R.Karlsson and R. Ståhlberg, Anal. Biochem., in press (1995)

Chapter 30. Catalytic RNA (Ribozymes) as Drugs

Nassim Usman and James A. McSwiggen
Ribozyme Pharmaceuticals, Inc.
Boulder, CO 80301

Introduction - The discovery of catalytic RNA (*i.e.* ribozymes) by Cech (1,2), and Altman (3), has fundamentally changed our view of the function of RNA in chemistry and biology. RNA traditionally has been viewed as a passive molecule that only carries information or provides structure. It is now clear that RNA can act as a catalyst, not only in RNA splicing and cleavage, but also in the cleavage of DNA (4) and peptide bonds, (5) and can also act as a kinase in the transfer of a phosphate group (6). The ability of catalytic RNA to carry out processes previously associated only with protein enzymes has caused a rethinking of the role that RNA may have played in early evolution (7).

The predominant activity found in naturally-occurring ribozymes is the ability to splice or cleave RNA molecules in a sequence-specific manner. Sequence specificity results from the base-pairing of ribozyme sequences with nucleotides near the cleavage site of the target RNA. Ribozymes function in an intramolecular (*cis*) reaction to splice or cleave their own RNA sequence, and they can also function in *trans* to cleave another RNA molecule. Because of their sequence specificity, ribozymes show promise as therapeutic agents to down-regulate a given RNA species in the background of cellular RNAs (8-11). Specifically, the mRNA coding for a protein associated with a disease state may be selectively cleaved as shown in Figure 1. This cleavage event renders the mRNA untranslatable and attenuates the protein's expression.

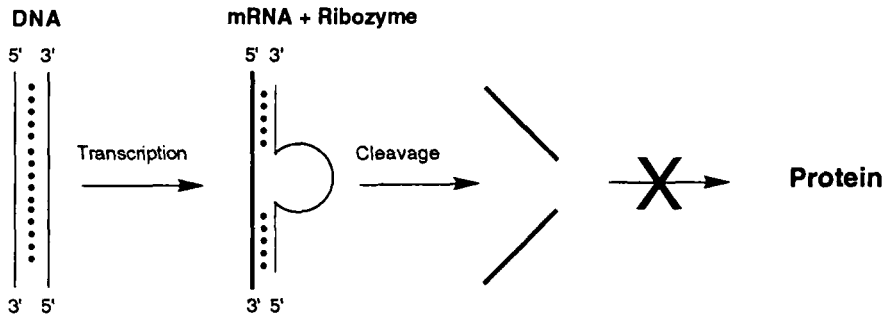


Figure 1: mRNA Cleavage by a Ribozyme

To function as therapeutic agents, ribozymes must (a) be deliverable to the target organs and cells, and (b) be able to destroy the target RNA in a time that is short relative to the natural half-life of that RNA. Delivery of ribozymes to the target cells can be accomplished either through exogenous drug delivery approaches or through gene therapy approaches, with each approach raising different issues for ribozyme delivery, stability, activity and potential toxicity. This review outlines the progress in utilizing ribozymes as human therapeutic agents. Readers interested in the basic science of ribozymes should also consult some recently published reviews of the field (12-18).

TYPES OF RIBOZYMES

Ribozymes have been found in a variety of motifs, locations and sizes. At least seven different ribozyme motifs have been identified in nature, including group I and group II introns, RNAse P RNA (M1 RNA), and *Neurospora* VS RNA, as well as the smaller hammerhead, hairpin, and HDV ribozymes (see Table 1 and Figure 2). In addition, at least three other RNA/protein complexes (ribosomes, splicosomes, and telomerase) are suspected of harboring ribozymes (19,20), and more ribozymes have been created through *in vitro* selection (21-23). While all of these ribozymes have the potential of being used as therapeutic agents, thus far only the naturally-occurring ribozymes have been considered in this light.

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Table 1 and Figure 2 summarize the main features of the naturally-occurring ribozyme motifs. These ribozymes differ in their size, structure, substrate specificity, mechanism of action, and potential suitability to therapeutic applications. Four ribozyme motifs (group I and group II introns, RNAse P RNA, and *Neurospora* VS RNA) fall into the category of "large" ribozymes, having sequences that exceed 150 nucleotides (nt). The large size of these ribozymes makes them somewhat less attractive as exogenous drug delivery candidates, but their size is not an issue for vector-mediated delivery.

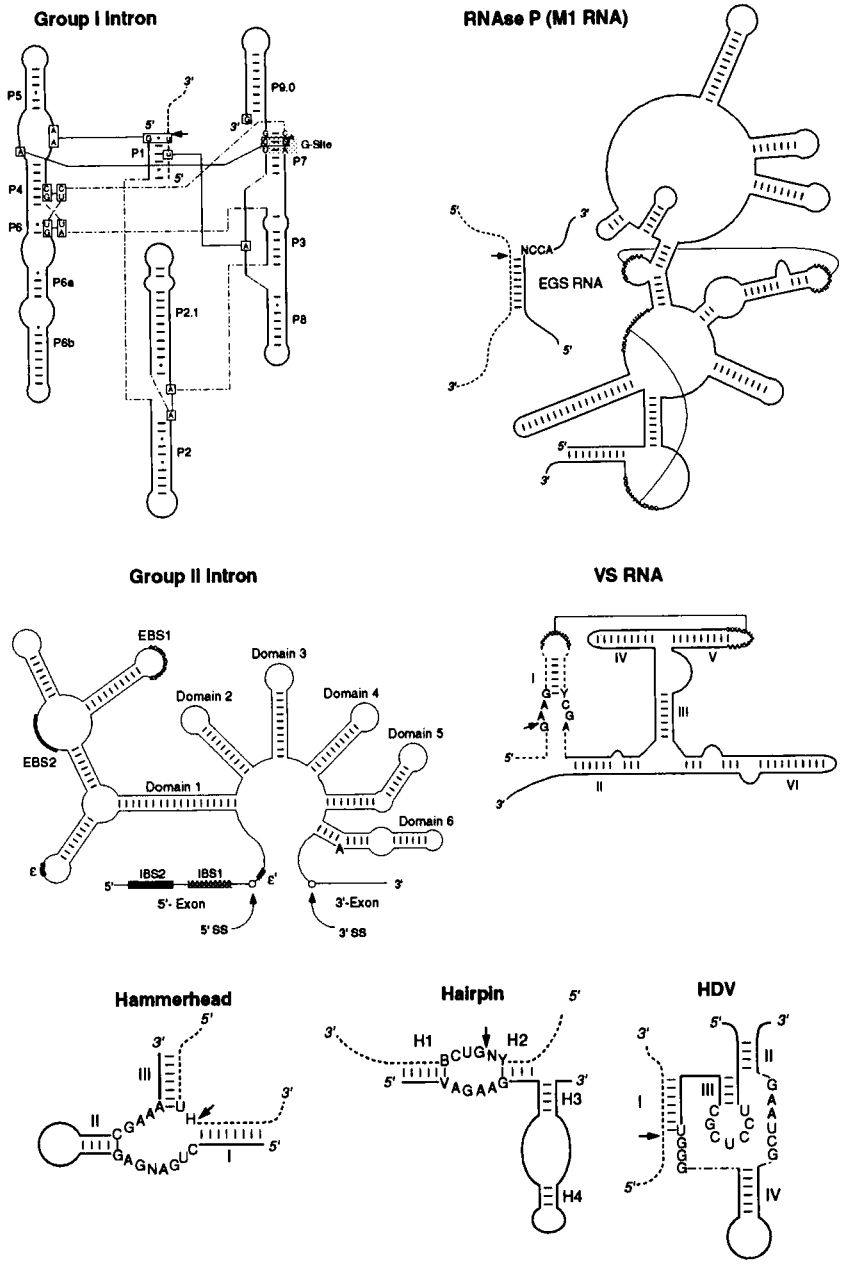


Figure 2: Structural Features of Naturally-occurring Ribozymes

The remaining three ribozyme motifs (hammerhead, hairpin, and HDV) vary in size from 13 to 80 nt depending upon the motif and where the division is made between enzyme and substrate. The most frequent arrangement in the hammerhead motif between ribozyme and substrate results in a ribozyme of roughly 35 nt. The hairpin and HDV ribozymes are a bit larger at roughly 60 nt. The small size of the hammerhead ribozyme makes it a clear favorite for exogenous ribozyme delivery protocols, however all three of these ribozymes are small enough to be considered for exogenous delivery.

Table 1: Characteristics of naturally occurring ribozymes

Group I Introns

- Size: ~200 to >1000 nucleotides.
- Requires a U in the target sequence immediately 5' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site.
- Reaction mechanism: attack by the 3'-OH of G to generate cleavage products with 3'-OH and 5'-G.
- Many group I introns are self-splicing, but others require additional protein cofactors presumably to help the intron fold into and/or maintain its active structure (24).
- Over 300 known members of this class. Found as an intervening sequence in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.

RNAse P RNA (M1 RNA)

- Size: ~290 to 400 nucleotides.
- RNA portion of a ubiquitous ribonucleoprotein enzyme.
- Cleaves tRNA precursors to form mature tRNA.
- Reaction mechanism: attack by M^{2+}-OH to generate cleavage products with 3'-OH and 5'-phosphate.
- RNAse P is found throughout the prokaryotes and eukaryotes. The RNA subunit has been sequenced from bacteria, yeast, rodents, and primates.

Group II Introns

- Size: >1000 nucleotides.
- Cleavage of target RNAs recently demonstrated.
- Sequence requirements not fully determined.
- Reaction mechanism: 2'-OH of an internal adenosine generates cleavage products with 3'-OH and a "lariat" RNA containing a 3'-5' and a 2'-5' branch point.

***Neurospora* VS RNA**

- Size: ~144 nucleotides.
- Cleavage of target RNAs recently demonstrated.
- Sequence requirements not fully determined.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Binding sites and structural requirements not fully determined.
- Only 1 known member of this class. Found in Neurospora VS RNA.

Hammerhead (HH) Ribozyme

- Size: ~13 to 40 nucleotides.
- Requires the target sequence UH immediately 5' of the cleavage site.
- Binds a variable number nucleotides on both sides of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent.

Hairpin (HP) Ribozyme

- Size: ~50 nucleotides.
- Requires the target sequence GUC immediately 3' of the cleavage site.
- Binds 4-6 nt at the 5'-side of the cleavage site and a variable number to the 3'-side of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 3 known members of this class. Found in three plant pathogen (satellite RNAs of the tobacco ringspot virus, arabis mosaic virus and chicory yellow mottle virus) which uses RNA as the infectious agent.

Hepatitis Delta Virus (HDV) Ribozyme

- Size: ~60 nucleotides.
- Cleavage of target RNAs recently demonstrated.
- Sequence requirements not fully determined.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Binding sites and structural requirements not fully determined; no sequences 5' of cleavage site are required.
- Only 2 known members of this class. Found in human HDV.

With the exception of RNAse P RNA, all naturally-occurring ribozymes act in *cis* to cleave or splice the RNA in which they are embedded. These *cis*-acting ribozymes can be made to function in *trans* by removal of the internal substrate followed by the addition of exogenous substrate (25). RNAse P is a *trans*-acting enzyme in nature, responsible for removing the 5'-leader from tRNA precursors to form mature tRNA (26).

Substrate Requirements and Reaction Mechanism - As Table 1 shows, the various ribozyme motifs differ greatly in their substrate requirements and reaction mechanisms. For the *Tetrahymena* group I intron, a U residue is required in the substrate 5' to the cleavage site, with binding determined by base-pairing interactions between substrate sequences upstream of the U and complementary sequences in the remaining 5 nt of the IGS. Substrate specificity is not strong for the *trans*-cleaving group I ribozymes, and as few as 2-3 base pairs (bp) complementarity between IGS and substrate can lead to cleavage (27,28). Thus, therapeutic applications for the group I ribozymes are likely to require improvements in substrate specificity.

All group I introns splice through a two step mechanism that involves, (a) cleavage at the 5'-splice junction with the concomitant addition of a non-encoded G nucleotide to the 5'-end of the intron, and (b) attack of the 5'-exon on the 3'-splice junction to produce ligated exons and free intron sequences (29). The cleavage and ligation steps in group I splicing are separable and reversible, so that a variety of therapeutic applications can be considered with these ribozymes. For example, in addition to targeting an RNA for cleavage, the *Tetrahymena* group I intron has been used to repair a "defective" β-galactosidase message by the ligation of new β-galactosidase sequences onto the defective message (30).

RNAse P (Figure 2) can be induced to cleave a target RNA by hybridizing an external guide sequence RNA (EGS) that allows the target to be recognized as a pre-tRNA destined for processing. For the bacterial RNAse P, cleavage can be achieved by hybridizing to the target RNA a short complementary RNA strand that is flanked by an unpaired NCCA sequence (31). The sequence requirements for the human RNAse P are more complicated; in that case the target and EGS must form a structure that more closely resembles the native tRNA structure (32). Thus, although the RNAse P ribozyme is too large for consideration as an exogenous delivery candidate, the possibility of activating the endogenous RNAse P could be explored. In contrast to the group I introns, RNAse P does not ligate RNAs, but only functions in cleaving its target RNA.

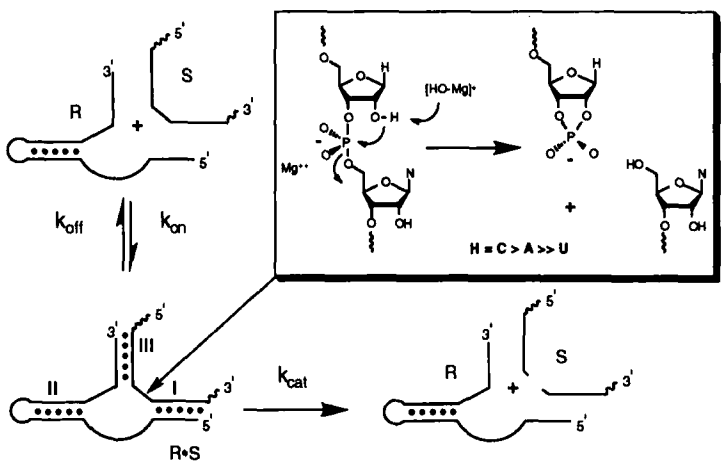


Figure 3: Base-specific Binding by the Arms of a Hammerhead Ribozyme

When divided in the way shown in Figures 2 and 3, hammerhead ribozymes require only the sequence UH immediately 5' to the cleavage site (where H = A, C, or U). In addition, sequences in Stems I and III of the hammerhead ribozymes must be complementary to the substrate sequences adjacent to the UH so that binding of ribozyme and substrate can occur. This requirement for the base-specific binding of the arms of the ribozyme to the substrate in the motif shown in Figure 3 is the basis for the rational design of hammerhead ribozymes to any target mRNA containing a UH site (8). In any RNA sequence the UH would be expected roughly once every 5 nucleotides. Generally, 5 or

more bp are required on each side of the cleavage site to achieve proper binding, however, substrate cleavage can be observed in situations where one substrate-binding arm contains as few as 2-3 bp as long as the other substrate-binding arm is tightly bound (33). The sequence requirements of the all-RNA catalytic core, has been determined and is shown in Figure 2 (34). Recently the detailed X-ray crystal structure of a hammerhead ribozyme has been solved (35).

As with the hammerhead ribozyme, the hairpin and HDV ribozymes form complementary base-pairing interactions between ribozyme and substrate sequences. The hairpin ribozyme binds to substrate sequences on both sides of the cleavage site, while the HDV ribozyme interacts only with the 3'-side of the substrate (Figure 2). The hairpin ribozyme has a somewhat more complicated substrate requirement, with the sequence CN↓GHYB required at the cleavage site (↓ indicates the cleavage site, and H = A, C, or U; Y = C or U; and B = C, G, or U)(36,37). In a random RNA sequence the hairpin cleavage site would be expected approximately once every 60 nucleotides.

The general mechanism of cleavage appears to be the same for hammerhead, hairpin, and HDV ribozymes, as well as for VS RNA, the most recently discovered natural ribozyme (38,39). These four ribozymes all form 2',3'-cyclic phosphate and 5'-OH termini in the cleavage products (Figure 3). The cleavage products are the same as are found in hydroxide catalyzed cleavage of RNA in which the 2'-OH of the ribose preceding the scissile bond acts as the nucleophile. The pH dependence of the cleavage reaction indicates that metal hydroxide plays a role in the reaction, possibly by abstracting hydrogen from the 2'-OH attacking group (40).

To date, almost all applications of ribozymes to the regulation of gene expression have used either the hammerhead or hairpin ribozymes. Thus, the remainder of this review discusses only these two types as drug candidates

SYNTHETIC RIBOZYMES

Unmodified RNA is unstable in biological sera. Therefore, a significant challenge in using ribozymes as drugs is to modify them chemically to increase their stability while retaining their catalytic activity. A number of structural modifications have been applied to oligonucleotides in general to enhance nuclease resistance (41-43). Improvements in the chemical synthesis of RNA (44,45) have expanded the ability to modify ribozymes. The majority of this work has been carried out with hammerhead ribozymes since their small size makes them amenable to chemical synthesis and modification (46,47). There are several positions in the basic nucleotide building blocks that may be modified. Some of these are shown in Figure 4. Specific examples of these types of modification and their effects on catalysis and nuclease resistance are described below.

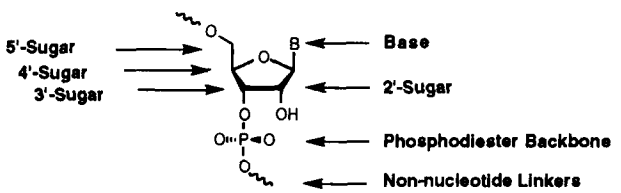


Figure 4: Nucleotide Modification Positions

2'-Sugar Modifications - Since the ribose 2'-hydroxyl group of RNA is its distinguishing feature, the first examples of ribozyme modification were conducted by simply removing the 2'-OH to achieve both nuclease resistance and to study the mechanism of catalysis in hammerhead ribozymes (48,49). Several reports on DNA/RNA chimeras have appeared in which the catalytic activity of the ribozymes is maintained (50,51).

In the case of 2'-substitutions, most have been carried out with 2'-*O*-alkyl, 2'-amino and 2'-fluoro replacements. For example, substitution of the 2'-*O*-Me modified nucleotide at all positions in a hammerhead ribozyme except G5, G8, A9, A15.1 and G15.2 resulted in a 1,000-fold increase in stability but lowered the catalytic activity significantly (52). In another investigation, a 2'-*O*-allyl-containing ribozyme with ribose residues at positions U4, G5, A6, G8, G12 and A15.1 retained 20% of the catalytic activity and increased the stability half-life, 30% of the material intact after 2 h compared to a <1 min half-life for the all-RNA ribozyme (53). Substitution of all the pyrimidine nucleotides in a hammerhead ribozyme by their 2'-amino or 2'-fluoro analogs resulted in a 1200-fold increased stability in rabbit serum, but also reduced activity 25-50-fold (54).

Specific substitutions of 2'-amino or fluoro adenosines or guanosines indicated that modification of core nucleotides generally reduced catalytic activity (55,56). To overcome this problem of balancing activity and catalytic activity, Beigelman *et al.* (57) constructed a generic nuclease stable hammerhead motif using selected modification of approximately thirty-two 2'-modified sugars, shown in Figure 5.

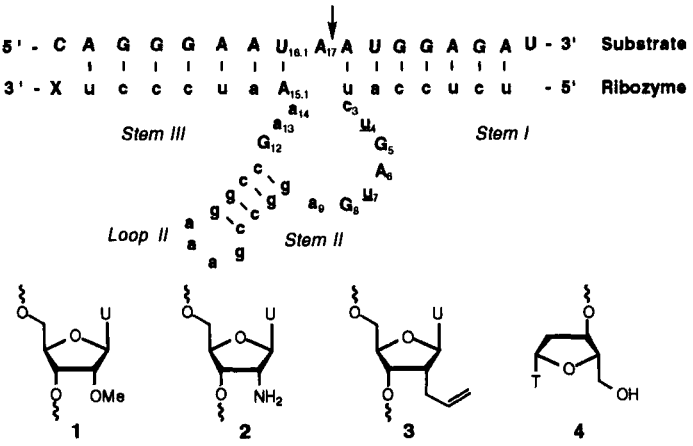


Figure 5: Generic Nuclease-stable Hammerhead Motif

Most of these were substituted as 2'-*O*-Me residues, **1** (lowercase in Figure 5). The remaining 2'-modifications were introduced at positions U4 or U7. The essential ribose residues in this model are located at the purine sites G5, A6, G8, G12 and A15.1 (uppercase in Figure 5). Two of these ribozymes, U4 and U7-2'-amino, **2**, and U4-2'-*C*-allyl, **3**, have wild-type catalytic activity and a 5-8 hour half-life in human serum at nM concentrations. A similar observation by Heidenreich *et al.* (58) in a perfluorinated ribozyme, showed that substitution of U4 and U7 by 2'-amino nucleotides rescued the catalytic activity to wild-type levels and maintained nuclease resistance. The catalytic activity of a ribozyme, is often expressed as the time required for 50% cleavage of a short substrate and is typically, for an all-RNA ribozyme, ~1-2 min. The addition of a 3'-3'-linked dT, **4**, (= X in Figure 5) or an abasic nucleoside to the ribozymes shown in Figure 5 increases their half-lives in serum to greater than 266 hours and had little effect on catalysis (57). This represents a 10^5-fold increase in ribozyme stability over the all-RNA ribozyme with only a negligible reduction in catalytic activity.

Phosphate Modifications - Phosphorothioates - The first study of phosphorothioate substitutions in hammerhead ribozymes was reported by Uhlenbeck's group (59). The P=S linkages were introduced enzymatically using T7 polymerase and resulted in the Rp diastereoisomer at each linkage. Four critical phosphates were identified at positions 5' to A9, A13, A14 and N18.1 (the cleavage site) where the Rp isomer significantly inhibited catalytic activity. This study indicated that the introduction of phosphorothioates in the catalytic core should be avoided at these specified positions. Shimayama *et al.* (60) showed that two phosphorothioate linkages 5' to U4 and G5, with replacement of U7 by adenosine or guanosine in a phosphorothioate-DNA/RNA chimera stabilized the molecule, but the catalytic activities of these chimeras were significantly reduced. The most successful use of phosphorothioates appears to be as stabilizing elements at the 5'- and 3'-ends of ribozymes in conjunction with core substitutions (58, *viz.* 2'-modifications above and base modifications below). In the end-stabilization case, no effect on catalysis has been noted.

Purine Base Modifications - Several modified bases have been incorporated into hammerhead ribozymes. Most of the substitutions have been made at purines in the core of the hammerhead. One of the first substitution studies used inosine (61-63) and purine ribonucleosides (61,62). In general, G-I changes had a significant effect (>20-fold decrease) on catalysis at positions G5 and G8. A-P changes at positions A6, A13 and A14 had little or no effect, although an A9-P9 replacement did reduce catalysis. Thus, it appears that the removal of the exocyclic 2-amino group from G is detrimental to catalysis while the removal of the 6-amino group from A, except in the case of A9, is relatively benign. Further purine substitutions with 2-aminopurine (63), xanthosine (63), and isoguanosine (63) also reduced catalysis in the case of G changes. To further probe the role of functional groups on G's in the catalytic core, O^6-methylguanosine (64) was incorporated. Once

again there was a significant detrimental effect seen at positions G5 and G8. N7-deazaadenosine (65,66), was used to probe the importance of N7-nitrogens of A residues in the core. In this case, the modification of A's was not detrimental to catalysis except at position A6. Finally, N7-deazaguanosine (67) was inserted at positions G8 and G12 with only a minor effect on catalysis. All of these purine replacement studies imply that potential non-canonical base-pairing interactions in the core are not important to catalysis while changes in the overall hydrogen bonding network, including H_2O and Mg^{2+}, are. To date none of the purine-base substituted ribozymes have been tested for stability against biological sera. It should be noted that the three purine bases most sensitive to modification, G5, A6 and G8 correspond to the positions at which 2'-OH's are required for efficient catalytic activity (52,53,57).

Pyrimidine Base Modifications - There have been two reports of pyrimidine analog substitutions in hammerhead ribozymes. In one case, C3 in the catalytic core was replaced with the fluorescent analog pyrimidine-2-one ribonucleoside (68) resulting in a moderate decrease in catalytic activity. In the other report, U4 and/or U7 were replaced by either 6-methyluridine (69) or by an abasic ribose (70), i.e. no base residue, in the "5-ribose" nuclease stable model (57). In both cases, the pyrimidine-base-modified ribozymes had wild-type catalytic activity. Furthermore, although these were ribonucleotide substitutions at endonuclease sensitive positions, the ribozymes were still stable to human serum.

Non-nucleotide Linkers - Since a number of modification studies (vide supra) have shown that Stem/Loop II of the hammerhead ribozyme is not required for catalytic activity, reports have appeared in which portions of the stem have been replaced with non-nucleotide linkers. Benseler et al. showed that hexaethylene glycol, triethylene glycol and propanediol linkers can be used to replace Stem/Loop II (71). Although some of the replacements did reduce catalytic activity, each type could, in the correct context of a stable Stem II, maintain catalysis. Similar results were reported for linkers of 7, 13, 17 and 19 atoms in length (72). The replacement of nucleotides by deoxyriboabasic and riboabasic linkers, sugars having no base (70), also had no effect on catalysis. These studies support the hypothesis that the majority of the Stem/Loop II region of the hammerhead ribozyme serves a general structural role in maintaining or allowing a certain conformation in the single-stranded catalytic core. There are no specific required base-base or base-metal interactions in this stem-loop. In a related area, studies have been carried out to remove Stem/Loop II entirely by deletion (73-75). The results indicate that this can be accomplished if the ribozyme is still able to fold correctly, but it is not a general phenomenon.

DELIVERY METHODS

As mentioned previously, ribozymes must be delivered to the target organs and cells to function as therapeutic agents. Three options exist for ribozyme delivery: direct exogenous delivery of synthetic ribozymes, exogenous delivery of a ribozyme expression vector with transient expression, and expression in cells stably transfected with a ribozyme expression vector. The two expression vector approaches offer the possibility of recruiting the cell to make ribozymes in the cellular compartments where it is needed, but this also limits the ribozyme synthesis to RNA only. Direct exogenous delivery of ribozymes permits the use of chemically synthesized (and nuclease stable) ribozymes, but this approach is likely to require higher and more frequent doses of ribozyme (since the cell does not participate in the synthesis). To date, most of the demonstrations of *in vivo* ribozyme efficacy have been carried out using ribozyme expression vectors, however efforts at exogenous ribozyme delivery are well under way and are beginning to show promise.

Exogenous Delivery - Small antisense oligos and ribozymes can be delivered free to cells. The uptake efficiency for free delivery is low, consequently the required oligo concentrations are high. This delivery method can be enhanced by the simple expedient of placing the ribozyme in a gel that will increase its effective concentration at the site of application, and will prevent it from being washed away from the target cells. This approach has been successfully employed to deliver antisense oligonucleotides to rat arteries (76). Exogenous delivery of ribozymes can be improved significantly by complexing or encapsulating the ribozyme with one of a variety of microcarriers. The microcarriers facilitate uptake by cells through endocytotic pathways. In addition, the microcarriers often protect the encapsulated oligonucleotides from nuclease degradation. Cationic lipids are one of the most well known microcarriers for oligonucleotide delivery. Cationic lipids bind to polynucleotides by virtue of their opposing charge. They have been used to deliver RNA, DNA, antisense oligonucleotides, and ribozymes (77-79). Cationic lipids also tend to be toxic to cells, so that variable results are often observed due to cell toxicity (80). The efficiency of ribozyme or polynucleotide uptake depends upon both cell type and phospholipid composition (81).

Expression Vectors - Expression vectors present both an opportunity and a challenge for ribozyme delivery (82). This arises from the fact that cells and viruses have evolved a variety of ways to deliver nucleic acid to specific cells and even specific compartments within the cell. The challenge comes in trying to determine which delivery mechanism is most suitable for the task.

Three different viral vectors are being examined as potential ribozyme delivery vehicles: retrovirus, adenovirus and adeno-associated virus (AAV). To date primarily RNA retroviruses have been used for ribozyme expression. The retroviruses infect a variety of cell types and stably integrate to afford the potential of long-term expression of ribozyme genes. However, integration of the retroviral genome requires cell division, so that many cell types are excluded from targeting by this virus. Adenovirus does not integrate into the host genome, and is thus attractive for situations where transient expression of ribozymes is desired. AAV is a non-pathogenic virus that requires the presence of helper virus (adenovirus) in order to replicate its genome. AAV can infect a broad range of cell types and does not require cell division for integration. These features make it attractive as a ribozyme expression vector.

Once the ribozyme gene is delivered to the cell it must still be expressed and allowed to find and cleave the target RNA. Expression of ribozymes can be directed from any of the eukaryotic promoters. The pol II promoters are used by cells primarily for the expression of messenger RNAs, which are then capped at the 5'-end, tailed with poly(A) at the 3'-end, and transported to the nucleus for translation. Ribozymes that are expressed from pol II promoters may be transported to the nucleus (which might be desirable), but may also be sequestered by proteins involved in transport and translation. Pol III promoters are used by cells to make a variety of smaller RNAs such as tRNA and the snRNAs. These RNAs experience a variety of different fates depending on their sequence and structure. Ribozymes expressed from pol III promoters may travel to the cytoplasm if they look like tRNAs, or they may be sequestered in the nucleus if they contain sequences marking them as snRNAs. Still more work is needed to determine the best way to express ribozymes *in vivo*.

BIOLOGICAL ACTIVITY

Ex Vivo Efficacy - Although the discovery of ribozymes is relatively recent, there have been a large number of reports of their ability to cleave mRNA in cell culture. One of the first reports was the demonstration of U7 snRNA cleavage in *Xenopus* oocytes (83). A hammerhead ribozyme was delivered to the oocytes by microinjection of a plasmid encoding the ribozyme between the A and B boxes of the $tRNA^{Met}$ gene. Another study in *Xenopus* oocytes (84) demonstrated the site specific cleavage of 28S rRNA in the α-sarcin domain by an *in vitro* transcribed ribozyme, and a concomitant reduction in protein synthesis expected from such a cleavage event. Other studies have shown ribozyme-mediated reduction of mRNAs of α-lactalbumin (85), tumor necrosis factor α (86) and pancreatic β-cell glucokinase (87) by vector encoded ribozymes. Chemically synthesized and stabilized ribozymes of the type described above (57) have reduced *c-myb* mRNA levels by ~90% in a variety of smooth muscle cells (88).

In the area of cancer research, a mammalian expression vector encoding a hammerhead ribozyme targeted to *c-fos* mRNA reduced gene expression of DNA synthesis enzymes and metallothionein (89). The ribozyme reduced *c-fos* mRNA and rendered *cis*-platin resistant cells susceptible to the anti-tumor drug, *cis*-platin. Another cancer target, the *bcr-abl* fusion gene (directly linked to Ph^1+ leukemia or chronic myelogenous leukemia, CML) was targeted using a vector-encoded hammerhead ribozyme. In a cell line derived from a patient with CML, the ribozyme reduced expression of both *bcr-abl* mRNA and its gene product, $p210^{bcr\text{-}abl}$ and inhibited cell growth (90). More recently chemically synthesized and stabilized ribozymes have been used to cleave *MDR-1* mRNA, the protein product of which is associated with multiple drug resistance, and restore chemosensitivity to resistant cells (91).

Viral diseases are also a potential target for ribozymes. In particular HIV has been the subject of several investigations using both hammerhead and hairpin ribozymes to destroy viral mRNA. Sarver *et al.* (9) used a mammalian expression vector encoding an anti-*gag* hammerhead ribozyme and showed a reduction of the *gag* mRNA and levels of the p24 soluble antigen. Cells transfected with a retroviral vector encoding a hairpin ribozyme targeted to the LTR of the HIV virus have shown resistance to infection following viral challenge (10,92). Another viral target, bovine leukemia virus, has also been targeted with ribozymes. A vector encoded hammerhead ribozyme was shown to reduce *rex/tax* mRNA levels by >80% in bat lung cells (93).

In Vivo Efficacy - There have been several reports of efficacy in animals to date. In a mouse model for bladder carcinomas, an anti-H-*ras* hammerhead ribozyme delivered to EJ cells *via* a vector, the rate of tumor invasion was decreased, and the survival rate increased 2-fold (94). In a study in *Drosophila*, transgenic eggs were generated that carry a ribozyme against the fushi tarazu (*ftz*) gene under the control of a heat-inducible promoter. This study distinguished the two developmental phases of the *ftz* gene, using timed heat-induction of the ribozyme.

Summary - In the life of ribozymes, significant strides have been made in taking a basic concept from discovery to potential drug candidates. It has been a little over 10 years since the first observation of catalysis by a large RNA in *cis*. It is now possible to design *trans*-cleaving ribozymes of several types, synthesize and stabilize them chemically, insert them into vectors as gene therapy agents and test them in an ever increasing number of cell and animal models. If this rate of progress continues, the therapeutic benefits of this class of drugs will be significant.

REFERENCES

1. T.R. Cech, A.J. Zaug and P.J. Grabowski, Cell, 29, 487 (1981).
2. K. Kruger, P.J. Grabowski, A.J. Zaug, J. Sands, D.F. Gottschling and T.R. Cech, Cell, 31, 147 (1982).
3. C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace and S. Altman, Cell, 35, 849 (1983).
4. A.A. Beaudry and G.F. Joyce, Science, 257, 635 (1992).
5. X. Dai, A. De Mesmaeker and G.F. Joyce, Science, 267, 237 (1995).
6. J.R. Lorsch and J.W. Szostak, Nature, 371, 31 (1994).
7. T.R. Cech, Gene, 135, 33 (1993).
8. J. Haseloff and W.L. Gerlach, Nature, 334, 585 (1988).
9. N. Sarver, E.M. Cantin, P.S. Chang, J.A. Zaia, P.A. Ladne, D.A. Stephens and J.J. Rossi, Science, 247, 1222 (1990).
10. J. Ojwang, A. Hampel, D. Looney, F. Wong-Staal and J. Rapapport, Proc.Natl.Acad.Sci.USA, 89, 10802 (1992).
11. B.A. Sullenger and T.R. Cech, Science, 262, 1566 (1993).
12. D. Castanotto, J.J. Rossi and N. Sarver, Adv.Pharmacol., 25, 289 (1994).
13. R.H. Symons, Curr.Opin.Struct.Biol., 4, 322 (1994).
14. T.R. Cech, Curr.Opin.Struct.Biol., 2, 605 (1992).
15. A.M. Pyle, Science, 261, 709 (1993).
16. D.M. Long and O.C. Uhlenbeck, FASEB J., 7, 25 (1993).
17. J. Bratty, P. Chartrand, G. Ferbeyre and R. Cedergren, Biochem.Biophys.Acta, 1216, 345 (1993).
18. M.D. Been, Cold Spring Harbor Monogr., 25, 407 (1993).
19. H.F. Noller, V. Hoffarth and L. Zimniak, Science, 256, 1416 (1992).
20. P.A. Sharp, Science, 254, 663 (1991),.
21. G.F. Joyce, Pure Appl.Chem., 65, 1205 (1993).
22. D.P. Bartel and J.W. Szostak, Science, 261, 1411 (1993).
23. T. Pan and O.C. Uhlenbeck, Biochemistry, 31, 3887 (1992).
24. G. Mohr, M.G. Caprara, Q. Guo and A.M. Lambowitz, Nature, 370, 147 (1994).
25. A.J. Zaug and T.R. Cech, Science, 231, 470 (1986).
26. H.D. Robertson,S. Altman and J.D. Smith, J.Biol.Chem., 247, 5243 (1972).
27. P.S. Kay and T. Inoue, Nature, 327, 343 (1987).
28. F.L. Murphy and T.R. Cech, Proc.Natl.Acad.Sci.USA, 86, 9218 (1989).
29. T.R. Cech, Science, 236, 1532 (1987).
30. B.A. Sullenger and T.R. Cech, Nature, 371, 619 (1994).
31. A.C. Forster and S. Altman, Science, 249, 783 (1990).
32. Y. Yuan, E.S. Hwang and S. Altman, Proc.Natl.Acad.Sci.USA, 89, 8006 (1992).
33. K.J. Hertel, D. Herschlag and O.C. Uhlenbeck, Biochemistry, 33, 3374 (1994).
34. D.E. Ruffner, G.D. Storm and O.C. Uhlenbeck, Biochemistry, 29, 10695 (1990).
35. H.W. Pley, K.M. Flaherty and D.B. McKay, Nature, 372, 68 (1994).
36. A. Hampel, R. Tritz, M. Hicks and P. Cruz, Nucleic Acids Res., 18, 299 (1990).
37. A. Berzal-Herranz, S. Joseph, B.M. Chowrira, S.E. Butcher and J.M. Burke, EMBO J. 12, 2567 (1993).
38. B.J. Saville and R.A. Collins, Cell, 61, 685 (1990).
39. H.C.T. Guo and R.A. Collins, EMBO J., 14, 368 (1995).
40. S.C. Dahm, W.B. Derrick and O.C. Uhlenbeck, Biochemistry, 32, 13040 (1993).
41. S.L. Beaucage and R.P. Iyer, Tetrahedron, 49, 6123 (1993).
42. J.F. Milligan, M.D. Matteucci and J.C. Martin, J.Med.Chem., 36, 1923 (1993).
43. E. Uhlmann and A. Peyman, Chem.Rev., 90, 543 (1990).
44. S.A. Scaringe, C. Franklyn and N. Usman, Nucleic Acids Res., 18, 5433 (1990).
45. F.W. Wincott, A. DiRenzo, C. Shaffer, S. Grimm, C. Workman, D. Sweedler and N. Usman, Nucleic Acids Res., in press (1995).
46. N. Usman and R. Cedergren, Trends Biochem. Sci., 17, 334 (1992).
47. O. Heidenreich, W. Pieken and F. Eckstein, FASEB J., 7, 90 (1993).
48. J.-P. Perreault, T. Wu, B. Cousineau, K.K. Ogilvie and R.J. Cedergren, Nature, 344, 565 (1990).
49. J.-P. Perreault, D. Labuda, N. Usman, J.-H. Yang and R.J. Cedergren, Biochemistry, 30, 4020 (1991).
50. N.R. Taylor, B.E. Kaplan, P. Swiderski, H. Li and J.J. Rossi, Nucleic Acids Res., 20, 4559 (1992).
51. S. Sawata, T. Shimayama, M. Komiyama, P.K.R. Kumar and K. Taira, Nucleic Acids Res., 21, 5656 (1993).
52. J.-H. Yang, N. Usman, P. Chartrand and R.J. Cedergren, Biochemistry, 31, 5005 (1992).
53. G. Paolella, B.S. Sproat and A.I. Lamond, EMBO J., 11, 1913 (1992).
54. W.A. Pieken, D.B. Olsen, F. Benseler, H. Aurup and F. Eckstein, Science, 253, 314 (1991).

55. D.M. Williams, W.A. Pieken and F. Eckstein, Proc.Natl.Acad.Sci.USA, 89, 918 (1992).
56. D.B. Olsen, F. Benseler, H. Aurup, W.A. Pieken and F. Eckstein, Biochemistry, 30, 9735 (1991).
57. L. Beigelman, J. McSwiggen, K. Draper, C. Gonzalez, K. Jensen, A. Karpeisky, A. Modak, J. Matulic-Adamic, A. DiRenzo, P. Haeberli, D. Tracz, S. Grimm, F. Wincott and N. Usman, Biochemistry, in press, (1995).
58. O. Heidenreich, F. Benseler, A. Fahrenholz and F. Eckstein, J. Biol.Chem., 269, 2131 (1994).
59. D.E. Ruffner and O.C. Uhlenbeck, Nucleic Acids Res., 18, 6025 (1990).
60. T. Shimayama, F. Nishikawa, S. Nishikawa and K. Taira, Nucleic Acids Res., 21, 2605 (1993).
61. G. Slim and M.J. Gait, Biochem.Biophys.Res.Comm., 183, 605 (1992).
62. D.-J. Fu and L.W. McLaughlin, Proc.Natl.Acad.Sci.USA, 89, 3985 (1992).
63. T. Tuschl, M.M.P. Ng, W. Pieken, F. Benseler and F. Eckstein, Biochemistry, 32, 11658 (1993).
64. J.A. Grasby, P.J.G. Butler and M.J. Gait, Nucleic Acids Res., 21, 4444 (1993).
65. D.-J. Fu and L.W. McLaughlin, Biochemistry, 31, 10941 (1992).
66. F. Seela, K. Mersmann, J.A. Grasby and M.J. Gait, Helv.Chim.Acta, 76, 1809 (1993).
67. D.-J. Fu and L.W. McLaughlin, Biochemistry, 32, 10629 (1993).
68. C.J. Adams, J.B. Murray, J.R.P. Arnold and P.G. Stockley, Tet.Lett., 35, 1597 (1994).
69. L. Beigelman, A. Karpeisky and N. Usman, Nucleosides Nucleotides, in press (1995).
70. L. Beigelman, A. Karpeisky and N. Usman, Bioorg.Med.Chem.Lett., 4, 1715 (1994).
71. F. Benseler, D.-J. Fu, J. Ludwig and L.W. McLaughlin, J.Am.Chem.Soc., 115, 8483 (1993).
72. J.B. Thomson, T. Tuschl and F. Eckstein, Nucleic Acids Res., 21, 5600 (1993).
73. M.J. McCall, P. Hendry and P.A. Jennings, Proc.Natl.Acad.Sci.USA, 89, 5710 (1992).
74. T. Tuschl and F. Eckstein, Proc.Natl.Acad.Sci.USA, 90, 6991 (1993).
75. D.M. Long and O.C. Uhlenbeck, Proc.Natl.Acad.Sci.USA, 91, 6977 (1994).
76. M. Simons, E.R. Edelman, J.L. DeKeyser, R. Langer and R.D. Rosenberg, Nature, 359, 67 (1992).
77. R.W. Malone, P.L. Felgner and I.M. Verma, Proc.Natl.Acad.Sci.USA, 86, 6077 (1989).
78. C.F. Bennett, M.Y. Chiang, H. Chan, J.E. Shoemaker and C.K. Mirabelli, Mol. Pharmacol., 41, 1023 (1992).
79. R. Philip, D. Liggitt, M. Philip, P. Dazin and R. Debs, J.Biol.Chem., 268, 16087 (1993).
80. J. Felgner, F. Bennet and P.L. Felgner, Methods: A Companion to Methods in Enzymology, 5, 67 (1993).
81. H. San, Z.-H. Yang, V.J. Pompili, M.L. Jaffe, G.E. Plautz, L. Xu, J.H. Felgner, C.J. Wheeler, P.L. Felgner, X. Gao, L. Huang, D. Gordon, G.J. Nabel and E.G. Nabel, Human Gene Therapy, 4, 781 (1993).
82. J.D. Thompson, D. Macejak, L. Couture and D.T. Stinchcomb, Nature Medicine, 1, 277 (1995).
83. M. Cotten and M.L. Birnstiel, EMBO J., 8, 3861 (1989).
84. K. Saxena and E.J. Ackerman, J.Biol.Chem., 265, 17106 (1990).
85. P.J. L'Huillier, S.R. Davis and A.R. Bellamy, EMBO J., 11, 4411 (1992).
86. M. Sioud, J.B. Natvig and Ø. Førre, J.Molec.Biol., 223, 831 (1992).
87. S. Efrat, M. Leiser, Y.-J. Wu, D. Fusco-DeMane, O.A. Emran, M. Surana, T.L. Jetton, M.A. Magnuson, G. Weir and N. Fleischer, Proc.Natl.Acad.Sci.USA, 91, 2051 (1994).
88. D.T. Stinchcomb, T.C. Jarvis, J.D. Thompson, K. Kisich, J.A. McSwiggen, D. Macejak, L. Alby, J.K. Belknap, R.A. Majak, D.F. Ayers, T.L. McKenzie, P. Stecha, T. Woolf, F.E. Wincott, N. Usman, E. Nabel and J.J. Marr, J.Cell.Biochem. Abstr.Supp., 19A, 202 (1995).
89. K.J. Scanlon, L. Jiao, T. Funato, W.I. Wang, T. Tone, J.J. Rossi and M. Kashani-Sabet, Proc.Natl.Acad. Sci.USA, 88, 10591 (1991).
90. D.S. Snyder, Y. Wu, J.L. Wang, J.J. Rossi, P. Swiderski, B.E. Kaplan and S.J. Forman, Blood, 82, 600 (1993).
91. M. Keihntopf, M.A. Brach, T. Licht, S. Petschauer, L. Karawajew and H.F. Kirschning, EMBO J., 13, (1994).
92. M. Yu, J. Ojwang, O. Yamada. A. Hampel, J. Rapapport, D. Looney and F. Wong-Staal, Proc.Natl.Acad. Sci.USA, 90, 6340 (1993).
93. G.H. Cantor, T.F. McElwain, T.A. Birkebak and G.H. Palmer, Proc.Natl.Acad.Sci.USA, 90, 10932 (1993).
94. M. Kashani-Sabet, T. Funato, T. Tone, L. Jiao, E. Yoshida, B.I. Kashfinn, T. Shitara, A.M. Wu, J.G. Moreno, S.T. Traweek, T.E. Ahlering and K.J. Scanlon, Antisense Res.Dev., 2, 3 (1992).

SECTION VII. TRENDS AND PERSPECTIVES

Editor: James A. Bristol
Parke-Davis Pharmaceutical Research, Ann Arbor, MI 48105

Chapter 31. To Market, To Market - 1994

Xue-Min Cheng
Parke-Davis Pharmaceutical Research, Ann Arbor, MI 48105

New chemical entities (NCEs), including several genetically engineered products, introduced for human therapeutic use into the world market for the first time during 1994, totaled 44 (1). This is similar to the 43 new introductions in 1993 (2) and well above the 36 introduced in both 1992 (3) and 1991 (4).

Having been the most active market for the new pharmaceuticals for several years, Japan once again took a significant lead in 1994 with 24 NCE introductions, accounting for 55% of the total new launches. The United States was second with 9 NCEs and the United Kingdom held the third place with 4 NCEs. The remainder of new drugs were introduced in 6 other countries, namely, Sweden, the Netherlands, and Canada, each with 2 NCEs, and Italy, France, Germany, and Austria, each with 1 NCE. Japan was also the leader in the category of originators for the NCEs with an impressive number of 21 which is nearly half of the total. The United States, where 11 NCEs were originated, followed as the second place again. Germany was the third with 3 and the United Kingdom, Switzerland, the Netherlands, France with 2 each, and lastly, Austria had 1 new drug.

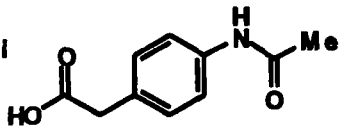
Following the trend from 1993, antiinfectives was the most active therapeutic category in terms of worldwide new launches. Eight new drugs including 4 antiviral agents and 2 carbapenems, reached the market for the first time. Antineoplastic and cardiovascular drugs were two other active therapeutic groups with 7 and 6 NCEs, respectively. CNS and antiinflammatory drugs followed with 5 NCEs each. Several new drugs in 1994 reflected real advances in the treatment of certain serious diseases. Highlights include donase alfa, the first new drug released in 30 years for cystic fibrosis, the most common among deadly genetic diseases in the United States; imiglucerase, the recombinant enzyme replacement product for type-1 Gaucher's disease; and sobuzoxane, the first available therapeutic agent for malignant lymphoma and adult T-cell leukemia/lymphoma (ATLL). Novel NCEs of special interest are exemplified by: losartan potassium, the first selective non-peptide angiotensin II (AT_1) antagonist for hypertension; the antiviral agent propagermanium, the first organogermanium compound marketed as a pharmaceutical; stavudine, a nucleoside analog for late-stage AIDS patients who are refractory to other therapies; venlafaxine, an antidepressant with novel dual serotonin/norepinephrine reuptake inhibitory activity.

In 1994, 22 new molecular entities were approved in the United States (5, 6), slightly less than the previous year (2). Among these, 12 (55%) were given the "priority" classification by the FDA, continuing the trend from 1993 that a higher percentage drugs are reviewed with "priority". There are 3 new approvals each in the therapeutic categories of pulmonary-allergy drugs and antivirals. Four of the 22 new approvals, famciclovir, imiglucerase, rocuronium bromide, and stavudine, reached their first worldwide market within the same year of their approvals. Noticeably, both the average and the median approval time for NCEs by the FDA decreased 33% from 1993 levels to 17.9 months and 15.5 months, respectively. The effect of user fees on the average approval time for NCEs was an even more significant reduction in review time to 12.6 months.

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Actarit (Antirheumatic) (7-9)

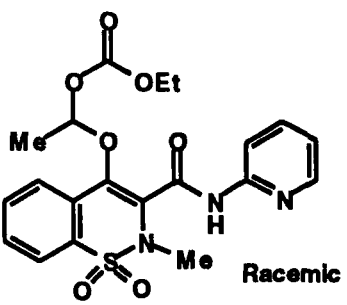
Country of Origin:	**Japan**
Originator:	**Nippon Shinyaku; Mitsubishi Kasei**
First Introduction:	**Japan**
Introduced by:	**Nippon Shinyaku; Mitsubishi Kasei; Nikken Chemicals**
Trade Name:	**Orcl; Mover**
CAS Registry No.:	**18699-02-0**
Molecular Weight:	**193.20**
Dosage Form:	**100 mg Tablet**



Actarit is an orally administered disease-modifying antirheumatic drug launched in Japan. Although its structure resembles that of non-steroidal anti-inflammatory drugs, it has no effect on experimental acute inflammation and has no analgesic or antipyretic effects. Its preventative and therapeutic effects on adjuvant arthritis are mediated via modulation of production and serum level of interleukin-2 which enhances production of suppressor T-cells to the immune system, thereby, preventing development of articular lesions.

Ampiroxicam (Antiinflammatory) (10,11)

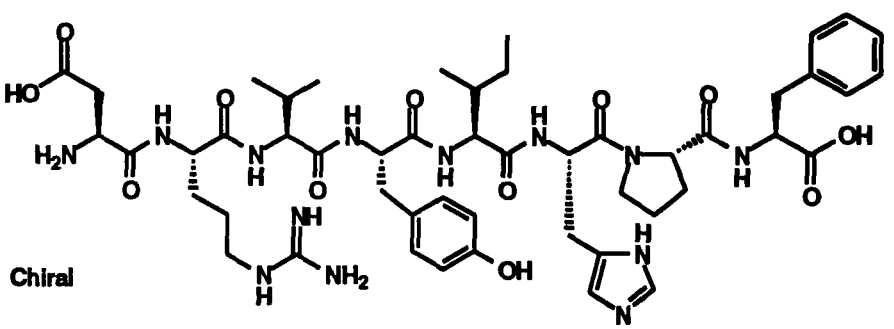
Country of Origin:	**Japan**
Originator:	**Pfizer**
First Introduction:	**Japan**
Introduced by:	**Pfizer; Toyama**
Trade Name:	**Nacyl; Flucam**
CAS Registry No.:	**99464-64-9**
Molecular Weight:	**447.46**
Dosage Form:	**13.5; 27 mg Capsule**



Racemic

Ampiroxicam is a once-daily non-steroidal anti-inflammatory drug (NSAID) introduced in Japan. As a non-acidic ether carbonate prodrug of the widely used antiinflammatory agent, piroxicam, ampiroxicam is similar or more potent than piroxicam in suppressing carrageenan edema, ultraviolet erythema, traumatic edema, capillary permeability, acetic acid writhing, yeast algesia and yeast hyperthermia in many animals. Bioavailability studies in man show undetectable plasma levels of ampiroxicam indicating 100% conversion to piroxicam during absorption process. Ampiroxicam has been reported to have fewer side effects on the gastric mucosa than piroxicam.

Angiotensin II (Anticancer Adjuvant) (12,13)



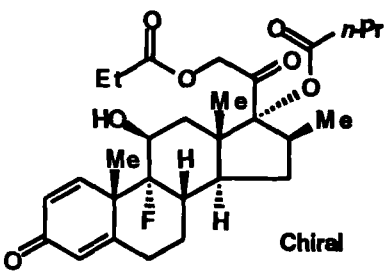
Chiral

Country of Origin:	**Japan**	Trade Name:	**Delivert**
Originator:	**Toa Eiyo**	CAS Registry No.:	**4474-91-3**
First Introduction:	**Japan**	Molecular Weight:	**1046.20**
Introduced by:	**Yamanouchi**	Dosage Form:	**0.05 mg Vial**

It is known that tumor tissues including metastatic lymph nodes are composed of newly growing vessels which lack blood flow autoregulation and are influenced only secondarily by the responding somatic vessels. Human angiotensin II (AT II), a vasopressor, was introduced in Japan to improve the efficacy of systemic chemotherapy. In patients with various advanced cancers, i.v. infusion of AT II to achieve a mean blood pressure of 1.5 times of baseline (but not over 150 mm Hg) followed by a bolus injection of conventional cytotoxic agents increases blood flow 5-7 fold in tumor tissues and therefore, increases drug delivery to the target tissue resulting in enhanced chemotherapeutic effects. This induced-hypertension chemotherapy regimen can be performed safely with minimal side effects.

Betamethasone Butyrate Propionate (Topical Antiinflammatory) (14)

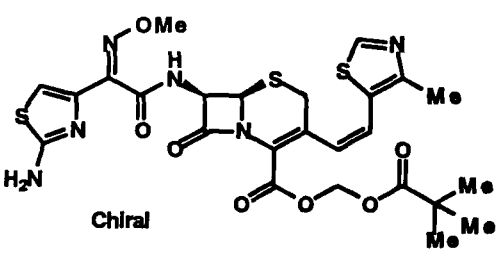
Country of Origin:	**Japan**
Originator:	**Mitsubishi Kasai**
First Introduction:	**Japan**
Introduced by:	**Torii**
Trade Name:	**Antebate; Antevate**
CAS Registry No.:	**5534-02-1**
Molecular Weight:	**518.63**
Dosage Form:	**0.05% Ointment cream**



Betamethasone butyrate propionate (BBP) was introduced in Japan last year as a topical antiinflammatory agent. It is a synthetic corticosteroid with strong antiinflammatory activity at the site of inflammation but without topical or systemic side effects. On paper disk granuloma in rats, BBP was shown to have higher degree of dissociation between antiinflammation efficacy and systemic side effects than other agents in this class. Antipyretic/analgesic properties have also been reported.

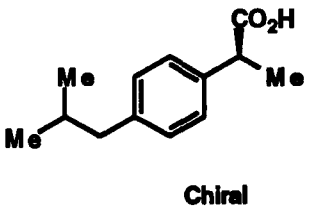
Cefditoren Pivoxil (Oral Cephalosporin) (15-17)

Country of Origin:	**Japan**
Originator:	**Meiji Seika**
First Introduction:	**Japan**
Introduced by:	**Meiji Seika**
Trade Name:	**Meiact**
CAS Registry No.:	**117467-28-4**
Molecular Weight:	**620.72**
Dosage Form:	**100 mg Tablet; Granule**



Cefditoren pivoxil is an orally active third generation cephalosporin introduced last year in Japan as a treatment for a broad range of bacterial infections including dermatological and other community acquired infections. Cefditoren pivoxil is reported to have a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, more potent than many other existing agents of its class. In particular, it shows the highest therapeutic activity against *S. pneumoniae* and *S. marcescens* infections. It exhibits resistance to β-lactamase hydrolysis typical of third generation cephalosporins. As a prodrug of cefditoren, it is readily absorbed through GI tract and has low toxicity and side effects.

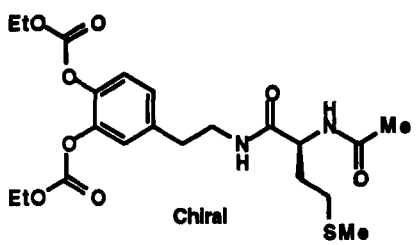
Dexibuprofen (Antiinflammatory) (18,19)



Country of Origin:	**Austria**
Originator:	**Gebro Broschek**
First Introduction:	**Austria**
Introduced by:	**Gebro Broschek**
Trade Name:	**Seractil**
CAS Registry No.:	**51146-56-6**
Molecular Weight:	**206.29**
Dosage Form:	**200; 300 mg Tablet**

Dexibuprofen, the *S*-(+)-isomer of the widely used NSAID agent ibuprofen, was launched in Austria for the treatment of rheumatoid arthritis. While the racemic compound is commonly used clinically, the antiinflammatory activity is mediated via the *S*-isomer by inhibition of prostaglandin synthesis. It has also been demonstrated that the *R*-isomer is converted to the *S*-antipode *in vivo* via a CoA thioester intermediate. Since CoA plays a pivotal role in intermediary metabolism and maintenance of the [acyl-CoA], generation of *R*-ibuprofen-CoA competitively inhibits many CoA-dependent reactions, which consequently produces perturbations of hepatocyte intermediary metabolism and mitocondrial function. Pure *S*-ibuprofen usage, therefore, is preferred allowing a reduction in dosage level and an improved side effect profile.

Docarpamine (Cardiostimulant) (20-22)



Country of Origin:	**Japan**
Originator:	**Tanabe Seiyaku**
First Introduction:	**Japan**
Introduced by:	**Tanabe Seiyaku**
Trade Name:	**Tanadopa**
CAS Registry No.:	**74639-40-0**
Molecular Weight:	**470.54**
Dosage Form:	**3 g Granule**

Docarpamine is a cardiostimulant with diuretic activity launched in Japan for the treatment of circulatory insufficiency. It is an orally effective peripheral dopamine prodrug that is well absorbed from the gastrointestinal tract and converted to dopamine *in vivo*, which exerts renal vasodilatory and natriuretic effects by activating renal dopamine (DA_1) receptors, and a positive inotropic effect by activating cardiac β_1-receptors. It has been reported to be effective in treating patients with acute heart failure, shock and acute renal failure.

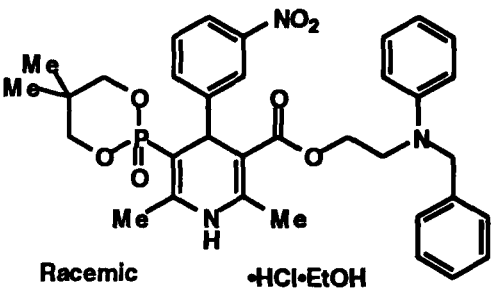
Dornase alfa (Cystic Fibrosis) (23-25)

Country of Origin:	**U.S.A.**	Trade Name:	**Pulmozyme**
Originator:	**Genentech**	CAS Registry No.:	**143831-71-4**
First Introduction:	**U.S.A.**	Dosage Form:	**2.5mg/2.5ml Ampule for Inhalation**
Introduced by:	**Genentech**		

Dornase alfa, the highly purified solution of recombinant human deoxyribonuclease I (rhDNase) produced by genetic engineering technology, was launched in 1994 first in the U.S.A. and shortly after in 7 other countries as the first new drug released in 30 years for the treatment of cystic fibrosis. Dornase alfa is effective in liquefying secretions from the lungs of cystic fibrosis patients by cleaving the extracellular DNA in purulent sputum and therefore reducing the viscoelasticity of the secretion. It also lessens dyspnea, improves lung function, reduces cough severity and frequency, increases the perception of well-being for the patients with cystic fibrosis related symptoms, and has excellent safety profile. Significant benefits have been reported in trials for the treatment of chronic obstructive pulmonary disease and acute infectious exacerbation of chronic bronchitis.

Efonidipine Hydrochloride Ethanol (Antihypertensive) (26-29)

Country of Origin: **Japan**
Originator: **Nissan Chemical**
First Introduction: **Japan**
Introduced by: **Zeria**
Trade Name: **Landel**
CAS Registry No.: **111011-76-8**
Molecular Weight: **714.20**
Dosage Form: **10; 20 mg Tablet**

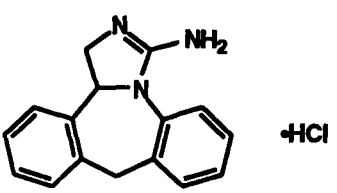


Racemic •HCl•EtOH

Efonidipine hydrochloride ethanol was first launched in Japan for the treatment of essential severe and renal hypertension. As the fourteenth dihydropyridine (DHP) calcium channel blocker to reach the market, it functions by binding to the DHP receptors on voltage-dependent calcium channels to cause diuretic and natriuretic actions in patients with essential hypertension. Its vasodilating and calcium antagonist effects are very slow in onset and long-lasting in all types of experimentally hypertensive models and the agent is reported to exhibit an improved side effect profile compared with other drugs of the same class. This may be explained by the slow and long-lasting inhibition of transmembrane Ca^{+2} uptake, which is accompanied by its very slow binding to and dissociation from the DHP receptors. A recent study in cholesterol-fed rabbits suggested that efonidipine may suppress the development of atherosclerosis without affecting the plasma lipids. Clinical trials for angina, pectoris, and for cerebrovasodilatory disorders have been reported.

Epinastine Hydrochloride (Antiallergy) (30-33)

Country of Origin: **Germany**
Originator: **Boehringer Ingelheim**
First Introduction: **Japan**
Introduced by: **Boehringer Ingelheim; Sankyo**
Trade Name: **Alesion**
CAS Registry No.: **80012-43-7**
Molecular Weight: **285.78**
Dosage Form: **10; 20 mg Tablet**



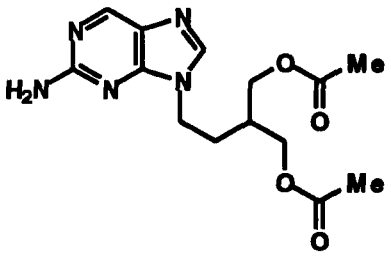
•HCl

Racemic

Epinastine hydrochloride, an orally active antihistaminic agent, was marketed in Japan for the treatment of bronchial asthma, allergic rhinitis, urticaria, eczema, dermatitis, and psoriasis vulgaris. Epinastine is one of the most effective peripherally acting histamine H_1-receptor antagonists without sedative effects. In addition, it exhibits potent anti-PAF and anti-LT activity which may also contribute significantly to its antiallergic activity. Chronic epinastine has been reported to effectively inhibit airway hyper-responsiveness in rats. Potent inhibitory effects of epinastine on bronchoconstriction induced by histamine and bradykinin, but not by other chemical mediators, has also been reported. Studies have indicated that there are no significant differences in pharmacological properties among *D*-, *L*-, and racemic epinastine.

Famciclovir (Antiviral) (34-37)

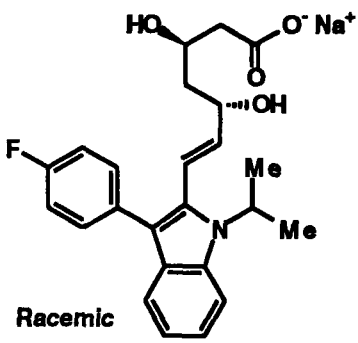
Country of Origin:	**United Kingdom**
Originator:	**SmithKline Beecham**
First Introduction:	**United Kingdom; U.S.A.**
Introduced by:	**SmithKline Beecham**
Trade Name:	**Famvir**
CAS Registry No.:	**104227-87-4**
Molecular Weight:	**321.34**
Dosage Form:	**250; 500 mg Tablet**



Famciclovir, an effective oral prodrug of the antiviral penciclovir, was launched in the UK and shortly after in the U.S.A. last year for the treatment of shingles (herpes zoster). As the diacetyl ester of the 6-deoxy- penciclovir, famciclovir has significantly improved oral absorption. It is converted to penciclovir *in vivo* with a bioavailability of 41% in rats. Penciclovir is phosphorylated, and hence activated only in herpesvirus infected cells. This stable metabolite, penciclovir-triphosphate, is a strong inhibitor of herpesvirus DNA polymerases and of viral DNA synthesis with long lasting effects. The high selectivity of famciclovir/penciclovir to herpes family of viruses and their rather low toxicity may make this agent superior to other existing drugs.

Fluvastatin Sodium (Hypolipaemic) (38-40)

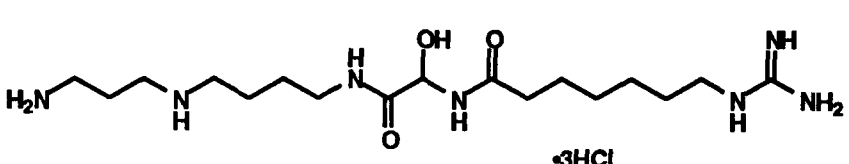
Country of Origin:	**Switzerland**
Originator:	**Sandoz**
First Introduction:	**United Kingdom**
Introduced by:	**Sandoz**
Trade Name:	**Lescol**
CAS Registry No.:	**93957-55-2**
Molecular Weight:	**433.46**
Dosage Form:	**20; 40 mg Capsule**



Racemic

Fluvastatin sodium is the fourth HMG-CoA reductase inhibitor to reach the market as a dietary adjunct for lowing total and low-density lipoprotein (LDL) cholesterol. Fluvastatin sodium is the first totally synthetic inhibitor of its class and has more potent effects on reducing serum total and LDL cholesterol and serum triglyceride levels than compactin or lovastatin. Moreover, fluvastatin exhibits a unique set of pharmacological properties including a biopharmaceutical profile most consistent with hepatoselectivity. It has been reported to be well tolerated and to exhibit a safety profile superior to other agents of its class. The main mechanism by which HMG-CoA reductase inhibitors lower plasma cholesterol has been suggested to be up-regulation of hepatocellular LDL-receptor expression and enhancement of receptor-mediated clearance and catabolism of LDL cholesterol.

Gusperimus Trihydrochloride (Immunosuppressant) (41-43)



•3HCl

Racemic

Country of Origin:	**Japan**	Trade Name:	**Spanidin**
Originator:	**Nippon Kayaku**	CAS Registry No.:	**104317-84-2**
First Introduction:	**Japan**	Molecular Weight:	**387.53**
Introduced by:	**Nippon Kayaku**	Dosage Form:	**100 mg Inj.**

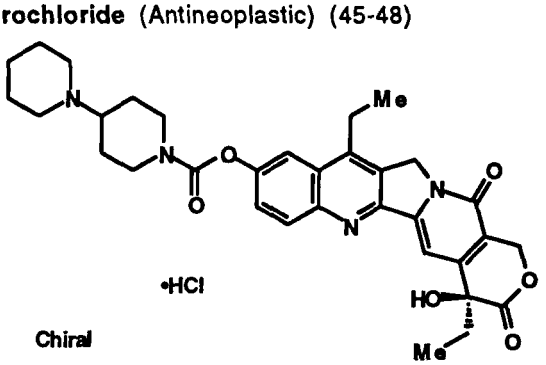
Gusperimus, a synthetic derivative of the antitumor antibiotic spergualin originally isolated from *Bacillus laterosporus*, was introduced in Japan as a treatment for accelerated and acute rejection reactions after kidney transplants. It possesses strong immunosuppressive activity including suppression of autoimmune disease, prolongation of survival of transplant allografts, induction of transplant tolerance, reversal of allograft rejection, inhibition of antibody responses, and suppression of delayed-type hypersensitivity. Although its activity is comparable to other agents as monotherapy, gusperimus in combination therapy (such as with cyclosporine A) has been reported to exhibit exceptional activity for prevention of transplant rejection and to induce long term tolerance. The mechanism of action appears to be due to inhibition of differentiation and proliferation of effector cells, including cytotoxic T cells and antibody producing B cells. Gusperimus is also in clinical trials for the treatment of graft rejection, multiple sclerosis, systemic lupus, diabetes mellitus, rheumatoid arthritis, nephrosis, and as an anticancer agent.

Imiglucerase (Type-1 Gaucher's Disease) (44)

Country of Origin:	**U.S.A.**	Trade Name:	**Cerezyme**
Originator:	**Genzyme**	CAS Registry No.:	**154248-97-2**
First Introduction:	**U.S.A.**	Dosage Form:	**200 units/vial**
Introduced by:	**Genzyme**		**for inj.**

Imiglucerase, a mannose-terminated form of human placental glucocerebrosidase produced by recombinant technology, was introduced for the treatment of type-1 Gaucher's disease. Imiglucerase catalyzes the hydrolysis of glucocerebroside and thus prevents accumulation of this lipid in organs and tissues. Compared with the natural enzyme, the modification by removal of the terminal sugars resulted in strong binding of the exposed mannose to lectins expressed on the surface of macrophages, which greatly increases the amount of enzyme taken up by the cells. In 6-month clinical trials, its unequivocal efficacy was shown by improvement in all patients with type-1 Gaucher's disease, leading to an increased hemoglobin levels and platelet counts, decreased incidence of epistaxis and bruising, reduced spleen and liver sizes, and improved skeletal parameters. This enzyme replacement therapy is well tolerated with few mild adverse reactions.

Irinotecan Hydrochloride (Antineoplastic) (45-48)



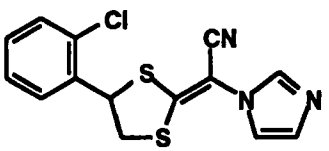
Chiral

Country of Origin:	**Japan**	Trade Name:	**Campto; Topotecin**
Originator:	**Yakult Honsha**	CAS Registry No.:	**100286-90-6**
First Introduction:	**Japan**	Molecular Weight:	**623.15**
Introduced by:	**Yakult Honsha; Daiichi Seiyaku**	Dosage Form:	**40 mg/2 ml; 100 mg/5 ml Inj.**

Irinotecan hydrochloride, a semi-synthetic, water soluble derivative of the potent anticancer agent camptothecin, was launched in Japan for the treatment of lung, ovarian, and cervical cancers. Irinotecan exerts its antitumor activity via inhibition of topoisomerase I, a cellular enzyme that is involved in maintaining the topographic structure of DNA during the process of translation, transcription, and mitosis. Irinotecan undergoes de-esterification *in vivo* to yield an active metabolite, SN-38, which is 1000-fold more potent than the parent. Although being much less toxic than camptothecin, a significant number of patients in clinical trials exhibited side effects of leukopenia, diarrhea, nausea/vomiting, and alopecia. Combination therapy of irinotecan with another widely used anticancer agent, cisplatin, has been reported to be superior to either agent alone. Irinotecan is in clinical trials for gastrointestinal, breast, skin, colorectal, pancreatic cancers, mesothelioma and non-Hodgkin's lymphoma.

Lanoconazole (Antifungal) (49,50)

Country of Origin: **Japan**
Originator: **Nihon Nohyaku**
First Introduction: **Japan**
Introduced by: **Nihon Nohyaku; Tsumura**
Trade Name: **Astat**
CAS Registry No.: **101530-10-3**
Molecular Weight: **319.83**
Dosage Form: **1% Cream and solution**

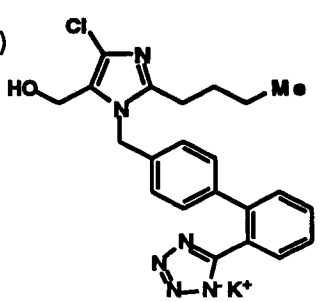


Racemic

Lanoconazole is a potent antifungal imidazole launched in Japan for the treatment of dermal mycoses. Lanoconazole is reported to be an inhibitor of ergosterol biosynthesis thus altering the ultra structure of the cell wall. It exhibits potent antifungal activity against a wide range of pathogenic fungi including yeast, dermatophytes, dermatiaceous fungi, dimorphic fungi, *Aspergillus* spp., *Penicillium* spp., and *Candida* spp., being particularly active against *Trichophyton* spp.. It is considerably more potent than other agents of the same family such as bifonazole and clotrimazole. Clinical efficacy was demonstrated by remarkable improvements in skin symptoms of the patients with tinea pedis, tinea corporis and cruis, candidiasis or tinea versicolor.

Losartan Potassium (Antihypertensive) (51-54)

Country of Origin: **U.S.A.**
Originator: **DuPont Merck**
First Introduction: **Sweden**
Introduced by: **DuPont Merck; Merck**
Trade Name: **Cozaar**
CAS Registry No.: **124750-99-8**
Molecular Weight: **461.01**
Dosage Form: **50 mg Tablet**

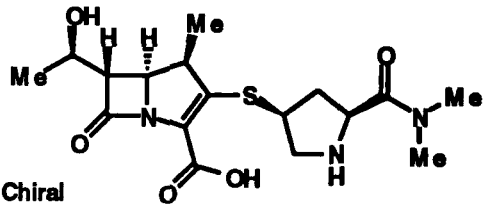


Losartan potassium is the first potent and selective non-peptide angiotensin II (AT II) AT_1 receptor antagonist introduced to the market as a once-daily oral antihypertensive. It is efficacious and long lasting in controlling blood pressure in spontaneous hypertensive rats, in patients with essential hypertension in addition to those patients with renal impairment. Since losartan functions by competitive antagonism at the level of receptor, which represents the most direct way of selectively inhibiting the renin-angiotensin system (RAS) independent of the source of AT II, its use has been reported to be free of the coughing side effect exhibited by the ACE inhibitors. In contrast to calcium channel blockers, losartan does not appear to cause ankle edema, headache and tachycardia. It is also reported to be in clinical trials for the treatment of heart failure. Other reports indicate that losartan may have potential efficacy as an anxiolytic, an antiglaucoma agent, in addition to in

providing protection against stroke and in preventing the myointimal proliferative response of the vascular wall after coronary angioplasty and surgery.

Meropenem (Carbapenem Antibiotic) (55-57)

Country of Origin:	**Japan**
Originator:	**Sumitomo**
First Introduction:	**Italy**
Introduced by:	**Zeneca**
Trade Name:	**Merrem**
CAS Registry No.:	**96036-03-2**
Molecular Weight:	**383.46**
Dosage Form:	**250;500; 1000 mg Inj.**

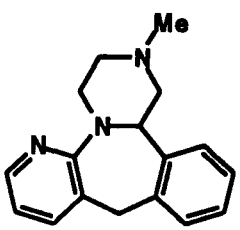


Chiral

Meropenem is a new carbapenem antibiotic introduced to market for the i.v. treatment of a wide variety of hospital infections such as lower respiratory tract, urinary tract, intraabdominal, gynecological and polymicrobial infections. Meropenem has a broad spectrum of antibacterial activity against most clinically important Gram-positive and Gram-negative aerobic and anaerobic bacteria with especially high potency against multiresistant *Enterobacteriaceae* and *Pseudomonas* aeruginosa. Compared with imipenem, the only other available carbapenem antibiotic, meropenem has the advantage of being dehydropeptidase 1 (DHP-1) stable and therefore does not need to be administered in conjunction with the DHP-1 inhibitor cilastatin. Meropenem is also being evaluated for treatment of resistant pseudomonal infections in cystic fibrosis patients.

Mirtazapine (Antidepressant) (58-60)

Country of Origin:	**Netherlands**
Originator:	**Organon**
First Introduction:	**Netherlands**
Introduced by:	**Organon**
Trade Name:	**Remeron**
CAS Registry No.:	**61337-67-5**
Molecular Weight:	**265.36**
Dosage Form:	**15; 30 mg Tablet**

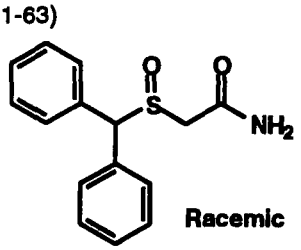


Racemic

Mirtazapine is an antidepressant launched in the Netherlands last year. Mirtazapine is a potent antagonist of presynaptic α_2 receptors as well as a moderately potent 5-HT antagonist. Studies suggested that blockade of α_2-adrenoceptors, but not inhibition of noradrenaline uptake, is involved in the mechanism of the antidepressant action of mirtazapine. Mirtazapine has demonstrated efficacy in various studies in depressed patients, being equal or more potent than mianserin, amitriptyline, etc. and exhibiting less anticholinergic and gastrointestinal side effects and low cardiovascular toxicity. In addition to its antidepressant effects, studies in animals indicated that mirtazapine has anxiolytic and/or hypnotic activity.

Modafinil (Narcolepsy and Idiopathic Hypersomnia) (61-63)

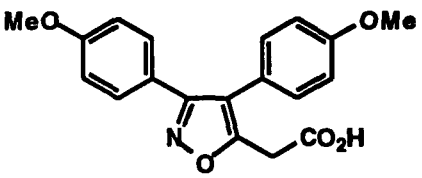
Country of Origin:	**France**
Originator:	**Lafon**
First Introduction:	**France**
Introduced by:	**Lafon**
Trade Name:	**Modiodal**
CAS Registry No.:	**68693-11-8**
Molecular Weight:	**273.35**
Dosage Form:	**100 mg Tablet**



Racemic

Modafinil, a centrally active α_1-adrenergic agonist, was marketed in France as a psychostimulant for the treatment of narcolepsy and idiopathic hypersomnia including Gelineau's syndrome. In monkeys, modafinil was found to induce potent behavioral stimulation and awakening without stereotyped behavior. In narcoleptic patients, modafinil reduces the daily number of sleep attacks significantly and markedly improves performance. The mechanism of action for its locomotor effects was reported to be due to the stimulation of the central α_1-adrenergic system, opposite to amphetamine and methylphenidate, which act mainly by dopaminergic mechanisms. Modafinil has been reported to exhibit minimal peripheral side effects at therapeutic doses and appears to have low abuse potential. The use of modafinil in the treatment of cerebral infarction and ischemia has been claimed.

Mofezolac (Analgesic) (64,65)



Country of Origin:	**France**
Originator:	**Pasteur Merieux**
First Introduction:	**Japan**
Introduced by:	**Taiho**
Trade Name:	**Disopain**
CAS Registry No.:	**78967-07-4**
Molecular Weight:	**339.35**
Dosage Form:	**75 mg Tablet**

Mofezolac is a new non-steroidal analgesic antiinflammatory agent introduced in Japan for the treatment of post-operative and post-traumatic pain, acute upper respiratory tract pain, osteoarthritis, and lumbago. In *in vitro* studies, mofezolac inhibits prostaglandin biosynthesis and platelet aggregation, reportedly through the inhibition of cyclooxygenase. In animal studies, mofezolac showed more potent suppression in various writhing models than agents such as ibuprofen, mefenamic acid and aspirin. Mofezolac also has a potent inhibitory activity on the algesic responses induced by the mechanical stimulus of the inflamed tissue. The ulcerogenic effect of mofezolac on the gastric mucosa is far weaker than that of indomethacin and other agents.

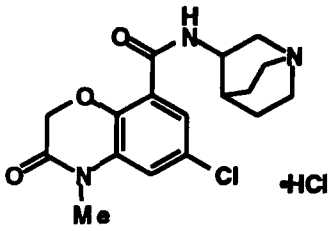
Nartograstim (Chemotherapy-Induced Leukopenia) (66-68)

Country of Origin:	**Japan**	Trade Name:	**Neu-Up**
Originator:	**Kyowa Hakko**	CAS Registry No.:	**134088-74-7**
First Introduction:	**Japan**	Dosage Form:	**50; 100; 250 µg/l**
Introduced by:	**Kyowa Hakko**		**Vial for inj.**

Nartograstim, a highly active mutein of the granulocyte colony-stimulating factor (G-CSF) produced by recombinant technology, was introduced in Japan for the treatment of chemotherapy-induced granulocytopenia, rhabdomyosarcoma and neutropenia associated with infantile acute lymphatic leukemia and infantile aplastic anemia. In mice, nartograstim showed an excellent accelerating effect on rapid recovery from severe leukopenia induced by typical anticancer agents such as mitomycin C and adriamycin. In patients with advanced lung cancer, nartograstim has been reported to produce a significant shortening of the period of neutropenia and an acceleration of neutrophil count recovery. With changes of five amino acids at the N-terminal region of human G-CSF, nartograstim has superior *in vivo* hematopoietic effects to the intact rhG-CSF due to its higher specific activity to progenitor cells and its improved physicochemical, biological and pharmacokinetic stability.

Nazasetron Hydrochloride (Antiemetic) (69-71)

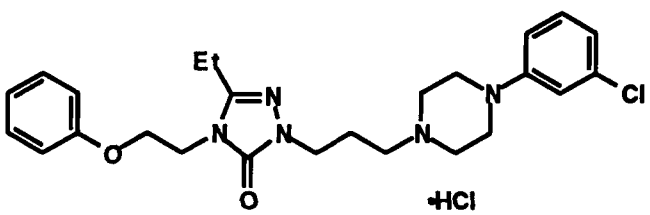
Country of Origin:	**Japan**
Originator:	**Yoshitomi**
First Introduction:	**Japan**
Introduced by:	**Yoshitomi; Japan Tabacco; Green Cross**
Trade Name:	**Serotone**
CAS Registry No.:	**123040-16-4**
Molecular Weight:	**386.28**
Dosage Form:	**10 mg/2 ml Ampule for inj.**



Racemic

Nazasetron (azasetron) hydrochloride is the fourth in the class of highly potent 5-HT_3 receptor antagonists to reach the market for prophylaxis of severe nausea and vomiting induced by cytotoxic chemotherapy and by total body X-radiation. In a study with 146 cancer patients suffering from emesis induced by cisplatin, nazasetron was reported to be markedly effective in 72% of subjects. Nazasetron is devoid of dopamine D_2 receptor blocking activity and therefore is free of the extrapyrimidal side effects associated with other commonly used antiemetics such as metoclopramide and domperidone. Nazasetron has been reported to be potentially useful for the treatment of gastrointestinal motility dysfunction, such as gastric stasis, vomiting and irritable bowel syndrome.

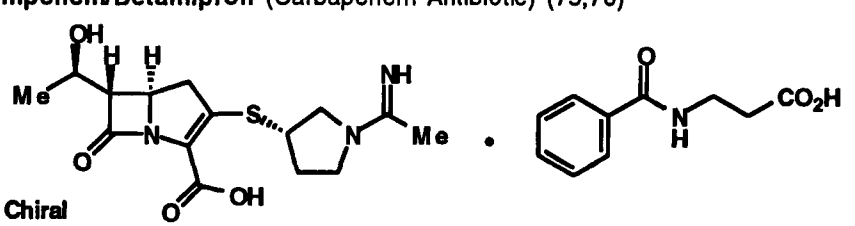
Nefazodone Hydrochloride (Antidepressant) (72-74)



Country of Origin:	**U.S.A.**		Trade Name:	**Serzone**
Originator:	**Bristol-Myers**	**Squibb**	CAS Registry No.:	**82752-99-6**
First Introduction:	**Canada**		Molecular Weight:	**506.48**
Introduced by:	**Bristol-Myers**	**Squibb**	Dosage Form:	**100; 150; 300 mg Tablet**

Nefazodone, a second generation trazodone type antidepressant, was launched last year in Canada. Nefazodone acts as both a potent 5-HT_2 receptor antagonist and as a serotonin (5-HT) reuptake inhibitor. This combination effect appears to enhance 5-HT_{1A}-mediated neurotransmission. Distinct from the first generation agents such as tricyclic antidepressants, nefazodone has very selective serotonergic effects with negligible affinity for cholinergic and histamine receptors, and low affinity for a_1-adrenergic receptors. Therefore, it has a superior toxicity profile that does not produce agitation, insomnia, and lacks cardiac side effects.

Panipenem/Betamipron (Carbapenem Antibiotic) (75,76)



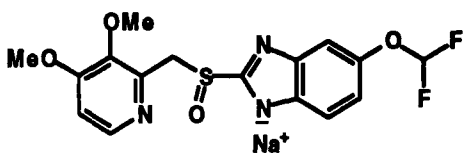
Chiral

Country of Origin:	**Japan**	Trade Name:	**Carbenin**
Originator:	**Sankyo**	CAS Registry No.:	**138240-65-0**
First Introduction:	**Japan**	Molecular Weight:	**532.62**
Introduced by:	**Sankyo**	Dosage Form:	**0.25; 0.5 mg Inj.**

Panipenem/betamipron was launched last year in Japan for the treatment of respiratory infections. The active ingredient in the mixture, panipenem, is a semisynthetic carbapenem that has potent antibacterial activity against a wide range of clinically important species of both Gram-positive and Gram-negative organisms including *Staph. aureus, E. faecalis* and *P. aeruginosa.*, being more effective than imipenem. Panipenem appears to be more efficacious *in vivo* than the estimated effect from MIC *in vitro*, particularly in experimental infections. Clinical studies indicated that panipenem is efficacious both in patients with severe infections complicating hematological disorders and in children where other antibiotics were ineffective. Betamipron, formulated with panipenem in a 1:1 fixed ratio, is an organic anion transport inhibitor to block the incorporation of panipenem into renal tubules, thus preventing renal dysfunction.

Pantoprazole Sodium (Antiulcerative) (77,78)

Country of Origin:	**Germany**
Originator:	**Byk Gulden**
First Introduction:	**Germany**
Introduced by:	**Byk Gulden; Schwarz Pharm**
Trade Name:	**Pantozol; Rifun**
CAS Registry No.:	**102625-70-7**
Molecular Weight:	**405.36**
Dosage Form:	**40 mg Tablet**



Racemic

Pantoprazole, an irreversible proton pump inhibitor, reached its first market worldwide in Germany for acute treatment of gastric and duodenal ulcers and gastroesophageal reflux disease. Proton pump inhibitors are more effective than other strategies in inhibiting acid secretion since they function at the final step of acid production, therefore, provide superior symptom relief and healing in all acid related diseases. As the third substituted benzimidazole proton pump inhibitor marketed, pantoprazole not only has superior ulcer-preventing effect but also is more potent than omeprazole in healing acetic acid-induced gastric and duodenal ulcers with extremely low acute toxicity. The mechanism of action for this class of compounds has been suggested to be mediated via the protonated form of the molecule which selectively reacts with cysteines present on the extracytoplasmic face of the enzyme to form covalent disulfide bonds.

Pegaspargase (Antineoplastic) (79,80)

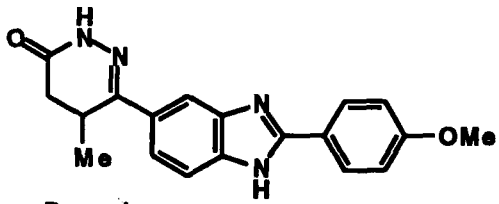
Country of Origin:	**U.S.A.**	Trade Name:	**Oncaspar**
Originator:	**Enzon**	CAS Registry No.:	**9015-68-3**
First Introduction:	**U.S.A.**	Dosage Form:	**5 ml Vial for Inj.**
Introduced by:	**Rhone-Poulenc Rorer**		

Pegaspargase, a polyethylene glycol conjugate of L-asparaginase (ASNase), was launched for combination chemotherapy in acute lymphoblastic leukemia (ALL). L-Asparaginase is an enzyme that inhibits protein synthesis by the depletion of sources of L-asparagine, which is necessary for transformed lymphoid cells to proliferate. It has been used as a standard component of the antileukemia armamentarium for childhood ALL. Pegaspargase has greater antitumor activity, a longer plasma half-life and less immunogenicity than ASNase. It produces minimal side effects after repeated dosing, whereas ASNase induces anaphylactic shock, urticaria, anorexia or vomiting and acute pancreatitis in dogs, and other immunological effects in man resulting from sensitization to the enzyme or protein

synthesis inhibition. The efficacy of pegaspargase for other indications including breast and lung cancers, non-Hodgkin's lymphoma and pancreatic cancer has been reported.

Pimobendan (Heart Failure) (81-83)

Country of Origin:	**Germany**
Originator:	**Boehringer Ingelheim**
First Introduction:	**Japan**
Introduced by:	**Boehringer Ingelheim**
Trade Name:	**Acardi**
CAS Registry No.:	**74150-27-9**
Molecular Weight:	**334.38**
Dosage Form:	**1.25;2.5mg Capsule**

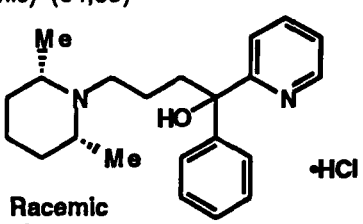


Racemic

Pimobendan, a novel cardiotonic vasodilator, was introduced in Japan for the treatment of acute and mild to moderate chronic heart failure. It is a phosphodiesterase III inhibitor and is able to enhance sensitization of myocardial contractile regulatory protein to calcium ions. The combination of these effects contribute to its inotropic activity. In patients with severe congestive heart failure, orally administered pimobendan improves cardiac index, stroke volume index, pulmonary wedge pressure, and systemic and pulmonary vascular resistance. Pimobendan is reported to be well tolerated and largely devoid of the proarrhythmic effects of classical phosphodiesterase III inhibitors. Studies also suggested that combination of the inotropic agent pimobendan with an ACE inhibitor such as enalapril may be superior to monotherapy for heart failure patients.

Pirmenol Hydrochloride (Antiarrhythmic) (84,85)

Country of Origin:	**U.S.A.**
Originator:	**Warner-Lambert**
First Introduction:	**Japan**
Introduced by:	**Warner-Lambert**
Trade Name:	**Pimenol**
CAS Registry No.:	**61477-94-9**
Molecular Weight:	**374.96**
Dosage Form:	**50; 100 mg Capsule**

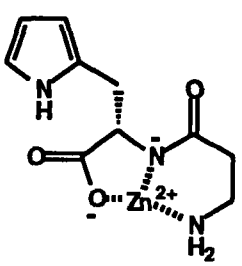


Racemic

Pirmenol hydrochloride is a long-lasting Class Ia antiarrhythmic agent introduced in Japan in 1994. The spectrum of antiarrhythmic activity of pirmenol includes atrial and ventricular arrhythmias of diverse etiology. In clinical trials, pirmenol was found effective in the short- and mid-term treatment of chronic premature ventricular beats, with or without underlying heart disease. In patients with supraventricular tachycardia, a single oral dose of pirmenol was highly effective in suppressing induction of tachycardia with adequate hemodynamic tolerance. Since pirmenol is highly effective by both intravenous and oral routes, it enables immediate suppression, prevention, or termination of cardiac arrhythmias and facilitates the transition to oral prophylactic therapy.

Polaprezinc (Antiulcerative) (86-89)

Country of Origin:	**Japan**
Originator:	**Hamari Chemicals**
First Introduction:	**Japan**
Introduced by:	**Zeria**
Trade Name:	**Promac**
CAS Registry No.:	**107667-60-7**
Molecular Weight:	**289.60**
Dosage Form:	**15%/g Granule**

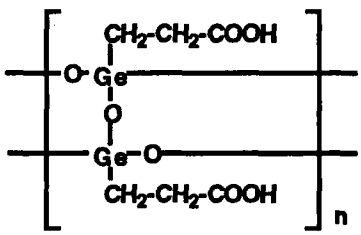


Chiral

Polaprezinc, a complex of zinc ion and *L*-carnosine, is an antiulcer agent launched in Japan. It is effective in promoting the healing of gastric ulcers in addition to preventing ulcer relapse. In rat studies, polaprezinc reduces the incidence of stomach lesions induced by ethanol. No effect was seen on ultrastructural damage or on cell proliferation and it appears that polaprezinc combines the cytoprotective effect of zinc with the ulcer healing properties of *L*-carnosine. The *in vivo* antioxidative properties of polaprezinc has been suggested to be responsible for its protective effect against gastric mucosal injury.

Recent studies revealed that polaprezinc is an activator of bone formation and has inhibitory effect of bone resorption. This agent is thus able to prevent the deterioration of bone metabolism indicating its potential usefulness in the treatment of osteoporosis. Polaprezinc has also been claimed for the prophylaxis and treatment of alcoholic hepatopathy, viral hepatitis or other hepatopathies caused by certain drugs, poisons and radiation.

Propagermanium (Antiviral) (90-93)



Country of Origin:	**Japan**
Originator:	**Sanwa Kagaku**
First Introduction:	**Japan**
Introduced by:	**Sanwa Kagaku; Yamanouchi**
Trade Name:	**Serosion**
CAS Registry No.:	**12758-40-6**
Dosage Form:	**10 mg Capsule**

Propagermanium, the first organogermanium compound marketed as a pharmaceutical, is indicated for the oral treatment of chronic hepatitis B. Propagermanium is reported to enhance production of interferon α/β in virus infected mice and in human peripheral blood mononuclear cells but does not inhibit the growth of various DNA or RNA viruses *in vitro*. Therefore, enhancement of the host immune system against virus infection was proposed to be responsible for its antiviral activity. Propagermanium also exhibits potent antitumor activity, reportedly via stimulation of T lymphocytes to produce a lymphokine (s), possibly interferon-γ, which induces the generation and activation of cytotoxic macrophages leading to elimination of tumor cells. Propagermanium has been reported to regulate calcium metabolism and to alleviate cancerous pain and osteocope.

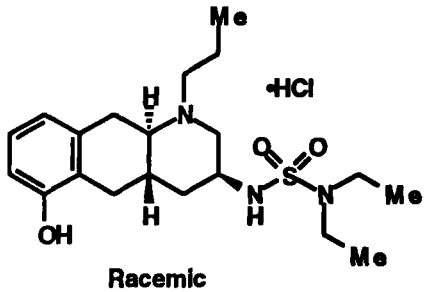
Pumactant (Lung Surfactant) (94,95)

Country of Origin:	**United Kingdom**	Introduced by:	**Britannia**
Originator:	**Britannia**	Trade Name:	**ALEC**
First Introduction:	**United Kingdom**	Dosage Form:	**100 mg Vial**

Pumactant, a mixture of dipalmitoylphosphatidylcholine (DPPC) and phosphatidylglycerol (PG) in the ratio of 7:3, is a high purity phospholipid based lung surfactant introduced in the United Kingdom for the prophylaxis and management of respiratory distress syndrome (RDS) in premature infants. Pumactant treatment to babies of 25 - 29 weeks gestation reduced the neonatal mortality from 27% to 14%, with a reduction of the severity of RDS and the incidence of parenchymal brain hemorrhage. It is reported that DPPC, the disaturated portion of phosphatidylcholine, accounts for the surface tension lowering ability and PG, which accumulates at the end of gestation, seems important at the onset of breathing. Pumactant is generally well tolerated and little side effects have been reported.

Quinagolide Hydrochloride (Hyperprolactinemia) (96-98)

Country of Origin:	**Switzerland**
Originator:	**Sandoz**
First Introduction:	**Netherlands**
Introduced by:	**Sandoz**
Trade Name:	**Norprolac**
CAS Registry No.:	**94424-50-7**
Molecular Weight:	**432.02**
Dosage Form:	**25;50;75;150 μg Tablet**

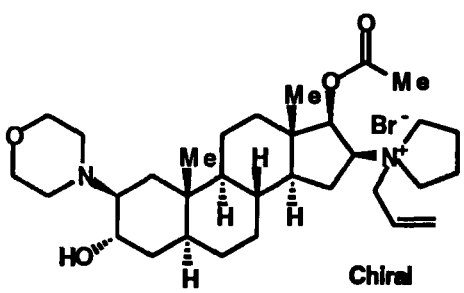


Racemic

Quinagolide is a potent and specific non-ergot dopamine D_2 agonist launched last year in the Netherlands for the treatment of hyperprolactinemia. Quinagolide is effective in inhibiting prolactin secretion by human pituitary tumor and hyperprolactinemia *in vitro* and *in vivo*. A daily dose of as low as 60 μg is able to achieve a pronounced and prolonged suppressive effect on serum prolactin and growth hormone secretion. It is also effective in providing relief from associated effects such as infertility, galactorrhea, oligomenorrhea, amenorrhea, loss of libido and impotence. Compared with the most widely used antiprolactin, bromocriptine, quinagolide is more potent *in vivo* with a longer duration of action and fewer side effects. A recent study showed that acute administration of quinagolide suppresses growth hormone secretion in acromegalic patients and thus could serve as an alternative therapy in acromegaly.

Rocuronium Bromide (Neuromuscular Blocker) (99,100)

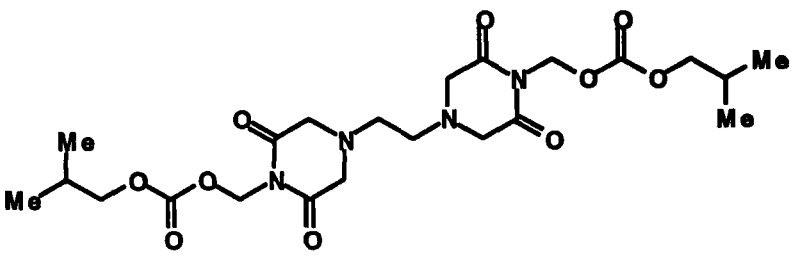
Country of Origin:	**Netherlands**
Originator:	**Organon**
First Introduction:	**U.S.A.; United Kingdom**
Introduced by:	**Organon**
Trade Name:	**Zemuron; Esmeron**
CAS Registry No.:	**119302-91-9**
Molecular Weight:	**609.69**
Dosage Form:	**10 mg/ml in 5 ml Vial**



Chiral

Rocuronium bromide is a short-acting nondepolarizing steroidal neuromuscular blocker introduced in the U.S.A. as Zemuron and in the United Kingdom as Esmeron. It is indicated for use as an adjunct to general anesthesia to facilitate endotracheal intubation and to provide skeletal muscle relaxation during surgery or mechanical ventilation. Rocuronium bromide has a significantly more rapid onset than other intermediate acting neuromuscular blockers, comparable to succinylcholine but with a very stable cardiovascular profile and has an improved side effect profile. It has been reported that good to excellent intubation conditions were achieved only one minute after a bolus dose of 600 μg/kg of rocuronium bromide. It has a short duration of action, therefore, is useful for rapid sequence intubation without unacceptable delays in the spontaneous recovery of neuromuscular function.

Sobuzoxane (Antineoplastic) (101-103)



Country of Origin:	**Japan**	Trade Name:	**Perazolin**
Originator:	**Zenyaku Kogyo**	CAS Registry No.:	**98631-95-9**
First Introduction:	**Japan**	Molecular Weight:	**514.53**
Introduced by:	**Zenyaku Kogyo**	Dosage Form:	**400; 800; 1200; 1600 mg Granule**

Sobuzoxane, a novel bis(2,6-dioxopiperazine) derivative, was introduced in Japan for the treatment of malignant lymphoma and adult T-cell leukemia/lymphoma (ATLL), which is resistant to chemotherapy and has a poor prognosis. Sobuzoxane exhibits a broad spectrum of antitumor activity. It is a noncleavable complex-stabilizing topoisomerase II inhibitor and exerts its function via interfering with the catalytic activity of the enzyme to result in an irreversible inhibition of cell division by blockade of chromosome condensation and segregation. Due to its unique mode of cytocidal activity, it has no cross resistance with many currently available anticancer agents. Sobuzoxane is reported to significantly inhibit growth of human colon, lung, gastric, and breast cancers and is in clinical trials for these indications.

Somatomedin-1 (Growth Hormone Insensitivity) (104,105)

Country of Origin:	**U.S.A.**	Trade Name:	**Igef**
Originator:	**Biogen**	CAS Registry No.:	**67763-96-6**
First Introduction:	**Sweden**	Molecular Weight:	**7649D**
Introduced by:	**Pharmacia**		

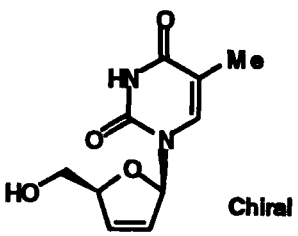
Somatomedin-1, an insulin-like growth factor-1, was launched last year in Sweden for the treatment of children with growth disorders caused by growth hormone insensitivity and for the treatment of a hereditary condition known as Laron-type dwarfism which is so far untreatable. Long-term administration of somatomedin-1 to these patients induced a rapid stimulation of linear growth both of the body and of the extremities. Somatomedin-1 has also been reported to play an important role in early brain development. As a hormone that resembles insulin in some respects, somatomedin-1 is a potent hypoglycemic agent and some studies have demonstrated the potential use of somatomedin-1 for treating diabetic patients. Somatomedin-1 is reportedly being investigated for the treatment of muscle atrophy, osteoporosis, and catabolism in critically ill patients.

Somatotropin (Growth Hormone) (106,107)

Country of Origin:	**U.S.A.**	Trade Name:	**Nutropin**
Originator:	**Genentech**	CAS Registry No.:	**12629-01-5**
First Introduction:	**U.S.A.**	Molecular Weight:	**~22 kD**
Introduced by:	**Genentech**	Dosage Form:	**5 mg/Vial; 10 mg/Vial for Inj.**

Somatotropin, a second-generation (methionine-free) human growth hormone produced by recombinant technology, was launched in the U.S.A. for the treatment of growth failure in children due to chronic renal insufficiency before transplantation and for the long term treatment of short stature in children with growth failure due to a lack of endogenous growth hormone secretion. The efficacy of somatotropin is reportedly comparable to the first biosynthetic growth hormone somatrem, which contains a methionyl residue, but with less frequent antibody formation and rarely causes allergic effects or impairs growth response. Somatotropin is also in clinical trials for the treatment of growth hormone deficiency in adults and is currently under approval review for Turner's syndrome.

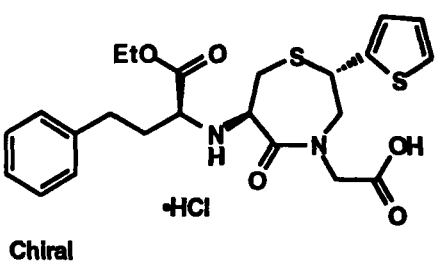
Stavudine (Antiviral) (108-110)



Country of Origin:	**U.S.A.**
Originator:	**Bristol-Myers Squibb**
First Introduction:	**U.S.A.**
Introduced by:	**Bristol-Myers Squibb**
Trade Name:	**Zerit**
CAS Registry No.:	**3056-17-5**
Molecular Weight:	**224.22**
Dosage Form:	**15; 20; 30; 40 mg Capsule**

Stavudine, a dideoxynucleoside analog of thymidine, has been introduced in the U.S.A. for the treatment of late-stage AIDS patients who are refractory to other AIDS treatments. Similar as other currently available agents for AIDS treatment such as zidovudine (AZT), didanosine, and zalcitabine, the anti-HIV activity of these 2'3'-dideoxynucleosides is ascribed to the inhibitory effect of their corresponding 5'-triphosphates against the HIV-encoded RNA-dependent DNA polymerase (reverse transcriptase). While some of these drugs have rapid development of drug resistance, stavudine is active against AZT-resistant HIV strains. It has a favorable pharmacokinetic profile with more complete and less variable oral absorption than AZT and didanosine and has a bioavailability of 80-90%.

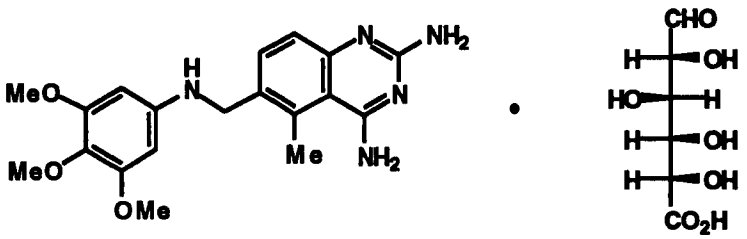
Temocapril Hydrochloride (Antihypertensive) (111-113)



Country of Origin:	**Japan**
Originator:	**Sankyo**
First Introduction:	**Japan**
Introduced by:	**Sankyo; Nippon Boehringer Ingelheim**
Trade Name:	**Acecol**
CAS Registry No.:	**110221-44-8**
Molecular Weight:	**513.07**
Dosage Form:	**1; 2; 4 mg Tablet**

Temocapril is a novel ACE inhibitor marketed in Japan for the treatment of hypertension. Temocapril is three times more potent than enalapril by oral administration, and has a long duration of action. It is characterized by a rapid onset of action resulting from a fast conversion of temocapril to the active metabolite diacid. Unlike most ACE inhibitors which are excreted mainly through the kidneys, temocapril is excreted predominantly through the biliary route indicating that temocapril is especially useful for treating hypertensive patients with renal dysfunction. The potential usefulness of temocapril in the treatment of congestive heart failure has been reported.

Trimetrexate Glucuronate (*Pneumocystis carinii* Pneumonia) (114-116)

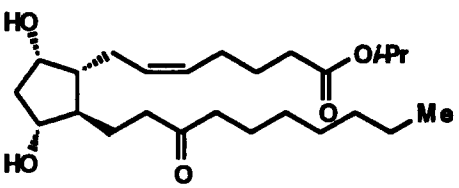


Country of Origin:	U.S.A.	Trade Name:	NeuTrexin
Originator:	Warner-Lambert	CAS Registry No.:	82952-64-5
First Introduction:	U.S.A.; Canada	Molecular Weight:	563.57
Introduced by:	U.S. Bioscience	Dosage Form:	25 mg Vial for Inj.

Trimetrexate glucuronate is a nonclassical antifolate introduced last year in the U.S.A. and Canada as second-line therapy for the treatment of *Pneumocystis carinii* pneumonia (PCP) and toxoplasmosis in AIDS patients who are refractory or intolerant to co-trimoxazole therapy. Trimetrexate glucuronate is reportedly to be in clinical trials for the treatment of several types of cancer including colorectal, gastric, pancreas, prostate, bladder, lung, breast, head, and neck cancers. It is also in early clinical trials for topical use in treating psoriasis and as an oral drug to treat rheumatoid arthritis. The mechanism of action of trimetrexate glucuronate is via inhibition of the enzyme dihydrofolate reductase which mediates the reduction of dihydrofolate to tetrahydrofolic acid and results in disruption of DNA, RNA and protein synthesis, with consequent cell death. Due to its toxicity to bone marrow and the GI tract, trimetrexate glucuronate must be concurrently administered with leucovorin as a rescue agent.

Unoprostone Isopropyl Ester (Antiglaucoma) (117,118)

Country of Origin:	Japan
Originator:	Ueno
First Introduction:	Japan
Introduced by:	Fujisawa
Trade Name:	Rescula
CAS Registry No.:	120373-24-2
Molecular Weight:	424.62
Dosage Form:	0.12%/ml Eye drops

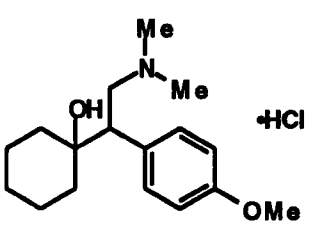


Chiral

Unoprostone isopropyl ester, a novel prostaglandin derivative, was launched last year in Japan as eye drops for the treatment of glaucoma and ocular hypertension. In animal and human studies, a single drop of unoprostone isopropyl ester induced significant decrease in intraocular pressure without systemic effects, myosis or mydriasis. Unlike prostaglandin analogs such as $PGF_{2\alpha}$, unoprostone isopropyl ester does not cause irritation in outer segment of the eye or significant changes in aqueous protein concentration and corneal endothelial permeability. Unoprostone isopropyl ester is suggested to act by increasing uveoscleral outflow or by decreasing episcleral venous pressure, resulting in ocular hypotension.

Venlafaxine Hydrochloride (Antidepressant) (119-121)

Country of Origin:	U.S.A.
Originator:	Wyeth-Ayerst
First Introduction:	U.S.A.
Introduced by:	Wyeth-Ayerst
Trade Name:	Effexor
CAS Registry No.:	99300-78-4
Molecular Weight:	313.87
Dosage Form:	12.5; 25; 37.5; 50; 75; 100 mg Tablet

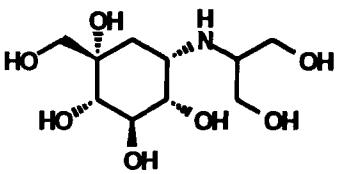


Racemic

Venlafaxine hydrochloride, a novel phenethylamine derivative, was introduced in the U.S.A. as an antidepressant. Venlafaxine is reported to be the first in the class of the second-generation of antidepressants with dual serotonin/norepinephrine reuptake inhibitory activity. Venlafaxine lacks any affinity for muscarinic, cholinergic, histaminergic and noradrenergic receptors and therefore, has an unusually favorable side effect profile compared with classical tricyclic antidepressants and shows less cardiotoxicity. In addition, venlafaxine has a rapid onset of action that makes it unique among the antidepressant agents. Other indications for venlafaxine include the treatment of obsessive and panic disorders, and obesity. Both enantiomers of venlafaxine were reported to have similar biological activity.

Voglibose (Antidiabetic) (122-124)

Country of Origin:	**Japan**
Originator:	**Takeda**
First Introduction:	**Japan**
Introduced by:	**Takeda**
Trade Name:	**Basen**
CAS Registry No.:	**83480-29-9**
Molecular Weight:	**267.28**
Dosage Form:	**0.2; 0.3 mg Tablet**



Chiral

Voglibose was introduced last year in Japan for the treatment of postprandial hyperglycemia in diabetic patients. As an orally active α-*D*-glucosidase inhibitor, it is effective in decreasing the release of glucose from carbohydrate ingestion and slowing the increase of postprandial blood glucose levels, as demonstrated in animals and humans following sucrose and starch loading. In addition, voglibose was reported to have prophylactic effect in genetically obese Wistar rats, maintaining low plasma levels of glucose, triglyceride and insulin, indicating a potential usefulness for the management of other carbohydrate-dependent metabolic disorders such as obesity. Voglibose is much more potent and has fewer side effects than acarbose, another agent in its class.

Zinostatin Stimalamer (Antineoplastic) (125-127)

Country of Origin:	**Japan**	Trade Name:	**SMANCS**
Originator:	**Yamanouchi**	CAS Registry No.:	**123760-07-6**
First Introduction:	**Japan**	Molecular Weight:	**~16000**
Introduced by:	**Yamanouchi; Kuraray**	Dosage Form:	**4; 6 mg Vial**

Zinostatin stimalamer is a new antineoplastic polymeric agent launched in Japan for the treatment of hepatoma. It was synthesized by chemical conjugation of the synthetic inert poly(styrene-maleic acid) (SMA) and neocarzinostatin (NCS), which is a proteinaceous antitumor agent produced by *Streptomyces carzinostaticus*. SMANCS not only retains the properties of NCS for inhibiting DNA synthesis in the cell by direct DNA strand scission, but also is able to uniquely augment the host defense system by inducing interferon γ and activating macrophages. Compared with NCS, SMANCS has greatly improved *in vitro* and *in vivo* stability, is more effective in reducing tumor size and in preventing the development of new tumors, has decreased toxic side effects and extended plasma half life. In clinical trials, tumor size reductions have been obtained in 90% of patients with hepatoma and dose of 0.7 mg/m^2 induced complete or almost complete necrosis of tumor without affecting noncancerous liver tissue or the gallbladder.

References

1. The material in this chapter is based on the combined information from the following sources:
   a. Scrip Magazine, January issue, (1995)
   b. Pharmaprojects
   c. IMSworld Publication
   d. J.R. Prous, DN&P, 8, 24 (1995).
2. X.-m. Cheng, Annu. Rep. Med. Chem., 29, 331 (1994).
3. J.D. Strupczewski and D.B. Ellis, Annu. Rep. Med. Chem., 28, 325 (1993).
4. J.D. Strupczewski and D.B. Ellis, Annu. Rep. Med. Chem., 27, 321 (1992).
5. F-D-C Reports, 5 (January 9, 1995).
6. W.M. Davis and M.C. Vinson, Drug Topics, 64 (February 6, 1994).
7. J.R. Prous, Ed., Drugs Future, 18, 752 (1993).
8. T. Nishimura, H. Fujisawa, H. Suzuka, H. Yoshifusa, Y. Nakamura, K. Inoue, Y. Shibata, K. Kimura, and M. Muramatsu, Jpn. Pharmacol. Ther., 21, 329 (1993).
9. H. Fujisawa, T. Nishimura, Y. Inoue, S. Ogaya, Y. Shibata, Y. Nakagawa, S. Sato, and K. Kimura, Arzneimittelforschung, 40, 693 (1990).
10. J.R. Prous, Ed., Drugs Future, 19, 586 (1994).
11. T.J. Carty, A. Marfat, F.F. Moore, F.C. Falkner, T.M. Twomey, and A. Weissman, Agents Actions, 39, 157 (1993).
12. H. Yamaue, H. Tanimura, S. Terashita, M. Iwahashi, M. Tani, T. Tsunoda, M. Tamai, and K. Mori, Arch. Jpn. Chir., 59, 302 (1990).
13. N. Tomura, T. Kato, I. Kanno, F. Shishido, A. Inugami, K. Uemura, S. Higano, H. Fujita, K. Mineura, and M. Kowada, Comput. Med. Imaging Graph., 17, 443 (1993).
14. Y. Koshiyama, Y. Maekawa, T. Kojima, K. Kariya, T. Muto, H. Ohtani, N. Ueda, S. Kanno, H. Uchiyama, M. Tamegaya, and M. Oda, Pharmacometrics, 47, 489 (1994).
15. J.R. Prous, Ed., Drugs Future, 18, 757 (1993).
16. S. Miyazaki, Y. Miyazaki, A. Tsuji, M. Nishida, and S. Goto, Antimicrob. Agents Chemother., 35, 1691 (1991).
17. D.H. Klaubert, J.M. Essery, and J.F. Barrett, Expert Opin. Invest. Drugs, 3, 133 (1994).
18. K.M. Knights and R. Drew, Biochem. Pharmacol., 44 , 1291 (1992).
19. K.P. Stock, G. Geisslinger, D. Loew, W.S. Beck, G.L. Bach, and K. Brune, Rheumatol. Int., 11, 199 (1991).
20. J.R. Prous, Ed., Drugs Future, 18, 174 (1993).
21. S. Nishiyama, M. Yoshikawa, and I. Yamaguchi, Cardiovasc. Drug. Rev., 10, 101 (1992).
22. C. Casagrande, L. Merlo, R. Ferrini, G. Miragoli, and C. Semeraro, J. Cardiovasc. Pharmacol. 14 (Suppl. 8), S40 (1989).
23. J.R. Prous, Ed., Drugs Future, 19, 542 (1994).
24. C. Gutteridge and R.J. Kuhn, Pediatr. Nurs., 20, 278 (1994).
25. J.D. Green, Hum. Exp. Toxicol., 13 (Suppl. 1), S1 (1994).
26. J.R. Prous, Ed., Drugs Future, 18, 760 (1993).
27. Y. Masuda, M. Takeguchi, C. Arakawa, T. Sakai, M. Hibi, S. Tanaka, K. Shigenobu, and Y. Kasuya, Arch. Int. Pharmacodyn. Ther., 304, 247 (1990).
28. T. Yokoyama, Y. Masuda, T. Sakai, S. Tanaka, and K. Tomita, J. Cardivasc. Pharmacol., 19, 851 (1992).
29. K. Toyoda, M. Kitahara, T. Yamashita, C. Shudo, Y. Masuda, M. Sakashita, S. Tanaka, and Y. Saito, Folia. Pharmacol. Jpn., 103, 231 (1994).
30. J.R. Prous, Ed., Drugs Future, 17, 1130 (1992).
31. K. Tasaka, M. Akagi, K. Izushi, and I. Aoki, Pharmacometrics, 39, 365 (1990).

32. J.C. Schilling, W.S. Adamus, and H. Kuthan, Int. J. Clin. Pharmacol. Ther. Toxicol., 28, 493 (1990).
33. K. Tasaka, C. Kamei, and S. Nakamura, Arzneimittelforschung, 44, 327 (1994).
34. J.R. Prous, Ed., Drugs Future, 19, 397 (1994).
35. R.A.Vere Hodge, Antiviral Chem. Chemother., 4, 67 (1993).
36. M.R. Boyd, S. Safrin, and E.R. Kern, Antiviral Chem. Chemother., 4 (Suppl. 1), 3 (1993).
37. D. Sutton and E.R. Kern, Antiviral Chem. Chemother., 4 (Suppl. 1), 37 (1993).
38. J.R. Prous, Ed., Drugs Future, 18, 862 (1993).
39. F. Guillot, P. Misslin, and M. Lemaire, J. Cardiovasc. Pharmacol., 21, 339 (1993)
40. L.A. Jokubaitis, Am. J. Cardiol., 73, 18D (1994).
41. J.R. Prous, Ed., Drugs Future, 19, 181 (1994).
42. M.A. Tepper, Ann. N. Y. Acad. Sci., 696, 123 (1993).
43. H, Jiang, S. Takahara, D. Li, M. Namiki, M. Ishibashi, A. Okuyama, and T. Sonoda, Transplant Proc., 26, 2313 (1994).
44. R. Whittington and K.L. Goa, Drugs, 44 , 72 (1992).
45. J.R. Prous, Ed., Drugs Future, 19, 295 (1994).
46. Y. Shimada, M. Rothenberg, S.G. Hilsenbeck, H.A. Burris, III, D. Degen, and D.D. Von Hoff, Anticancer Drugs, 5, 202 (1994).
47. H.A. Burris, III, and S.M. Fields, Hematol. Oncol. Clin. North Am., 8, 333 (1994).
48. N. Masuda, M. Fukuoka, S. Kudoh, Y. Kusanoki, K. Matsui, N. Takifuji, K. Nakagawa, M. Tamanoi, T. Nitta, T. Hirashima, S. Negoro, and M. Takada, Br. J. Cancer, 68, 777 (1993).
49. J.R. Prous, Ed., Drugs Future, 19, 300 (1994).
50. H. Oka, Y. Niwano, T. Ohmi, T. Tanaka, M. Uchida, and H. Yamaguchi, Arzneimittelforschung, 42, 345 (1992).
51. J.R. Prous, Ed., Drugs Future, 19, 407 (1994).
52. P.K. Siegl, J. Hypertens., 11 (Suppl. 3), S19 (1993).
53. P.B. Timmermans, D.J. Carini, A.T.Chiu, J.V. Duncia, W.A. Price, Jr., G.J. Wells, P.C. Wong, A.L. Johnson, and R.R. Wexler, Am. J. Hypertens., 4, 275S (1991).
54. S.S. Gottlieb, K. Dickstein, E. Fleck, J. Kostis, T.B. Levine, T. LeJetel, and M. DeKock, Circulation, 88, 1602 (1993).
55. J.R. Prous, Ed., Drugs Future, 19, 601 (1994).
56. R.D. Pryka and G.M. Haig, Ann. Pharmacother., 28, 1045 (1994)
57. U. Schumacher, B. Manncke, K. Gerbracht, and H. Werner, Arzneimittelforschung, 44, 859 (1994).
58. J.R. Prous, Ed., Drugs Future, 17, 1135 (1992).
59. W.T. Smith, V. Glaudin, J. Panagides, and E. Gilvary, Psychopharmacol. Bull., 26, 191 (1990).
60. T. DeBoer, F. Nefkens, and A. Van Helvoirt, Eur. J. Pharmacol., 253, 125 (1994).
61. J.R. Prous, Ed., Drugs Future, 19, 193 (1994).
62. T.J. Lyons and J. French, Aviat. Space Environ. Med., 62, 432 (1991).
63. F.A. Rambert, J. Pessonnier, and J. Duteil, Eur. J. Pharmacol., 183, 455 (1990).
64. J.R. Prous, Ed., Drugs Future, 18, 1162 (1993).
65. N. Ono, N. Yamamoto, A. Sunami, Y. Yamasaki, and H. Miyake, Nippon Yakurigaku Zasshi, 95, 63 (1990).
66. T. Suzuki, S. Tohda, K. Nagata, Y. Imai, I. Murohashi, and N. Nara, Acta. Haematol., 87, 181 (1992).
67. M. Okabe, M. Asano, K. Gomi, and T. Hirata, Jpn. Pharmacol. Ther., 19, 55 (1991).

68. M. Okabe, M. Asano, T. Kuga, Y. Komatsu, M. Yamasaki, Y. Yokoo, S. Itoh, M. Morimoto, and T. Oka, Blood, 75, 1788 (1990).
69. J.R. Prous, Ed., Drugs Future, 19, 283 (1994).
70. K. Haga, K. Inaba, H. Shoji, Y. Morimoto, T. Fukuda, and M. Setoguchi, Jpn. J. Pharmacol., 63, 377 (1993).
71. T. Fukuda, M. Setoguchi, K. Inaba, H. Shoji, and T. Tahara, Eur. J. Pharmacol. 196, 299 (1991).
72. J.R. Prous, Ed., Drugs Future, 18, 770 (1993).
73. R. Fontaine, Clin. Neuropharmacol., 16 (Suppl. 3), S45 (1993).
74. S.K. Hemrick-Luecke, H.D. Snoddy, and R.W. Fuller, Life Sci., 55, 479 (1994).
75. J.R. Prous, Ed., Drugs Future, 19, 631 (1994).
76. J. Shimada and Y. Kawahara, Drugs Exptl. Clin. Res., XX (6), 241 (1994).
77. J.R. Prous, Ed., Drugs Future, 15, 801 (1990).
78. J.M. Shin, M. Besancon, C. Prinz, A. Simon, and G. Sachs, Aliment. Pharmacol. Ther., 8 (Suppl. 1), 11 (1994).
79. E.G. MacEwen, R.C. Rosenthal, L.E. Fox, A.S. Loar, and I.D. Kurzman, J. Vet. Intern. Med., 6, 230 (1992).
80. M.J. Keating, R. Holmes, S. Lerner, and D.H. Ho, Leuk. Lymphoma., 10 (Suppl.), 153 (1993).
81. J.R. Prous, Ed., Drugs Future, 19, 700 (1994).
82. S.H. Kubo, Coron. Artery Dis., 5, 119 (1994).
83. A. Fitton and R.N. Brogden, Drugs & Aging, 4, 417 (1994).
84. J.R. Prous, Ed., Drugs Future, 18, 390 (1993).
85. Angiology, 39, 279 (1988).
86. J.R. Prous, Ed., Drugs Future, 18, 1165 (1993).
87. M. Yamaguchi and M. Hashizume, Peptides, 15, 633 (1994).
88. M. Yamaguchi, Y. Segawa, N. Shimokawa, N. Tsuzuike, and E. Tagashira, Pharmacology, 45, 292 (1992).
89 T. Yoshikawa, Y. Naito, T. Tanigawa, T. Yoneta, and M. Kondo, Biochim. Biophys. Acta. 1115, 15 (1991).
90. J.R. Prous, Ed., Drugs Future, 18, 905 (1993).
91. Y. Ishiwata, E. Suzuki, T. Otuka, S. Yokochi, F. Tasaka, H. Usuda, and T. Mitani, Pharmacometrics, 47, 155 (1994).
92. R.R. Brutkiewicz and F. Suzuki, In Vivo, 1, 189 (1987).
93. H. Fujita and Y. Seto, Pharmacometrics, 39, 385 (1990).
94. S.J. Hopkins, Drugs Today, 26, 445 (1990).
95. G.I. Martin and B.D. Sindel, Clin. Perinatol., 19, 461 (1992).
96. J.R. Prous, Ed., Drugs Future, 18, 973 (1993).
97. T. Nickelsen, E. Jungmann, P. Althoff, P.M. Schumm-Draeger, and K.H. Usadel, Arzneim. Forsch Drug Res., 43, 421 (1993).
98. T. Svoboda, H. Kotzmann, M. Clodi, P. Bernecker, G. Geyer, and A. Luger, Endocr. Res., 20, 59 (1994).
99. T.C. Wicks, AANA-J, 62, 33 (1994).
100. R.R. Bartkowski, T.A. Witkowski, S. Azad, J. Lessin, and A. Marr, Anesth. Analg., 77, 574 (1993).
101. J.R. Prous, Ed., Drugs Future, 19, 419 (1994).
102. R. Ohno, T. Masaoka, S. Shirakawa, S. Sakamoto, M. Hirano, S. Hanada, K. Yasunaga, S. Yokomaku, Y. Mitomo, K. Nagai, K. Yamada, and H. Furue, Cancer, 71, 2217 (1993).
103. T. Narita, Y. Koide, S-.I. Yaguchi, S. Kimura, Y. Izumisawa, M. Takase, M. Inaba, and S. Tsukagoshi, Cancer Chemother. Pharmacol. 28, 235 (1991).
104. Z. Laron, Drugs, 45, 1 (1993).
105. B.A. Scheven, N.J. Hamilton, T.M. Fakkeldij, and S.A. Duursma, Growth Regul., 1, 160 (1991).

106. S.D. Frasier, C.R. Rudlin, H.J. Zeisel, H.H. Liu, P.C. Long, B. Senior, D.N. Finegold, B.B. Bercu, J. F. Marks, and G.P. Redmond, Am. J. Dis. Child, 146, 582 (1992).
107. J.L. Johnson, M.T. Coffey, K.L. Esbenshade, B.R. Schricker, and D.H. Pilkington, J. Anim. Sci., 68, 3204 (1990).
108. J. Balzarini, Pharm. World Sci., 16, 113 (1994).
109. N. Clumeck, J. Antimicrob. Chemother., 32 (Suppl. A), 133 (1993).
110. K.M. Neuzil, Am. J. Med. Sci., 307, 368 (1994).
111. J.R. Prous, Ed., Drugs Future, 19, 421 (1994).
112. K. Oizumi, H. Koike, T. Sada, M. Miyamoto, H. Nishino, Y. Matsushita, Y. Iijima, and H. Yanagisawa, Jpn. J. Pharmacol., 48, 349 (1988).
113. S. Tokunaga, H. Oguchi, M. Higuchi, M. Tokoo, M. Miyasaka, K. Yazaki, H. Saito, H. Shioya, Y. Kawahara, and S. Furuta, Drug Invest., 7, 161 (1994).
114. C. Robinson, Drugs Today, 30, 347 (1994).
115. T.L. Levien and D.E. Baker, Hosp. Pharm., 29, 696 (1994).
116. J. Maroun, Semin. Oncol., 15, (Suppl. 2), 17 (1988).
117. J.R. Prous, Ed., Drugs Future, 19, 314 (1994).
118. M. Sakurai, M. Araie, T. Oshika, M. Mori, K. Masuda, R. Ueno, and M. Takase, Jpn. J. Ophthalmol., 35, 156 (1991).
119. J.R. Prous, Ed., Drugs Future, 18, 878 (1993).
120. Phase III Profiles, 3, 16 (1993).
121. J. Clin. Psychiatry, 54, 119 (1993).
122. J.R. Prous, Ed., Drugs Future, 18, 879 (1993).
123. T. Matsuo, H. Odaka, and H. Ikeda, Am. J. Clin. Nutr., 55 (Suppl.), 314S (1992).
124. H. Odaka, A. Shino, H. Ikeda, and T. Matsuo, J. Nutr. Sci. Vitaminol. 38, 27 (1992).
125. J.R. Prous, Ed., Drugs Future, 18, 880 (1993).
126. X. Rabasseda and N. Mealy, Drugs Today, 30, 221 (1994).
127. F. Suzuki, R.B. Polland, S. Uchimura, T. Munakata, and H. Maeda, Cancer Res., 50, 3897 (1990).

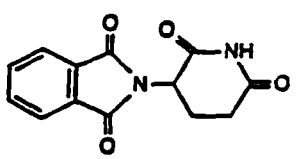
Chapter 32. Potential Use of Thalidomide in HIV/AIDS

David Stirling
Celgene Corporation
Warren, New Jersey 07059

Introduction - A newly recruited medical officer at the Food and Drug Administration (FDA) was given her first assignment in September of 1960 (1); Dr. Francis Kelsey was the medical reviewer on a new drug application (NDA) for thalidomide, submitted the previous month. At that time, marketing approval for new drugs was based upon the validity of safety claims alone and if there was no response from the agency within the 60 day review period, automatic market approval of the drug would follow.

Reports of peripheral neuritis plus other application deficiencies delayed approval of thalidomide, a new, highly regarded sedative. Ultimately, the agency was informed in November 1961 that the drug had been withdrawn from market in Germany because of reports linking the drug to an epidemic of birth defects. Thalidomide has never been approved for use in the U.S.

The current safety and efficacy regulations controlling approval of new drug applications in the U.S. and many other countries, can largely be attributed to the tragedies surrounding thalidomide 25 years ago. This was a major contribution of thalidomide to pharmaceutical development. Surprisingly other positive developments started to surface as early as 1965. Six leprosy patients exhibiting severe lepra reactions were given thalidomide as a sedative (2). There was a dramatic improvement in the inflammatory reaction of these patients, and since then thalidomide has become the drug of choice worldwide, including the U.S., for this inflammatory condition associated with leprosy, i.e. erythema nodosum leprosum (ENL). Despite the absence of any true understanding of the mechanism of action of the drug, it has been used with success in a number of refractory disease conditions e.g. Behçets syndrome, lupus erythematosus, oropharangeal ulcerations, graft-vs-host disease, ulcerative colitis, purigo nodularis (3, 4).



1

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

THALIDOMIDE CHEMISTRY

Thalidomide (α-(N-phthlalimido)glutarimide) pharmaceutical preparations (**1**) are racemic mixtures with a maximum solubility of 45-60 μg/ml (5). All four amide bonds of the molecule are susceptible to hydrolytic cleavage at a pH > 6 (5). Plasma pharmacokinetics were estimated in healthy male volunteers (6) using a single, 200 mg oral dose. The average mean peak concentration was 1.15 ± 0.2 μg/ml with absorption and elimination half-lives of 1.70 ± 1.05 hours and 8.70 ± 4.11 hours, respectively. The primary route of elimination is thought to be non-renal. Rates of elimination for the single enantiomers *in vivo* were recently estimated as 0.079 hr^{-1} for (R)-thalidomide and 0.24 hr^{-1} for (S)-thalidomide (7).

A number of biological activities have been attributed to thalidomide in addition to the sedative and teratogenic effects, e.g. peripheral neuropathy (5), inhibition of tumor necrosis factor α (TNFα) (8) and inhibition of angiogenesis (9). It is unclear if any of these activities is associated with either one or both of the enantiomers of thalidomide.

Most literature on the importance of chiral discrimination has concerned the teratogenic effect and has been contradictory in nature. However, most chemistry and biochemistry textbooks discussing chirality are replete with the omnipresent thalidomide stereochemistry example (S-teratogen, R-non-teratogen). An early report using separately synthesized enantiomers administered orally showed little difference in teratogenic potential in rabbits (10). Chromatographically separated optical isomers when administered intraperitoneally to mice and rats demonstrated dose-dependent teratogenicity with the (S)- enantiomer only (11).

Attempts to assay the biological activities of the single enantiomers are rendered somewhat meaningless if it can be demonstrated that administration of a single enantiomer is followed by rapid racemisation *in vivo*. Such studies have recently been facilitated with the development of reliable, high performance liquid chromatography (HPLC) assays applicable to serum samples of thalidomide (7, 12). Rapid interconversion was observed in human volunteers (7). Mean rate constants for chiral inversion for the (+)-(R)-thalidomide and (-)-(S)-thalidomide in blood at 37°C were estimated at 0.17 and 0.12 hr^{-1}, respectively (7). Hence any postulated structure/activity relationship associated with (R) or (S) thalidomide for either therapeutic or adverse effect is essentially redundant *in vivo*.

HIV/AIDS

Approaches to Therapy - Human Immunodeficiency Virus - 1 (HIV), the etiological agent responsible for Acquired ImmunoDeficiency Syndrome (AIDS) was first isolated in 1983. Less than three years later the results of a trial evaluating a novel, anti-retroviral drug Azidothymidine, (AZT) became public

(13). Much hope was based on these early, promising results but almost ten years later the picture is much gloomier. After AZT approval, the main focus of clinical research for HIV infection centered on designing improved, nucleoside-reverse transcriptase inhibitors like AZT and compounds directed at other essential viral functions. For example, protease inhibitors, tat antagonists and various non-nucleoside-reverse transcriptase inhibitors have all been developed. However, during this time the limitation of therapeutic efficacy for both the currently approved anti-retroviral drugs as well as others in development, have become apparent (14-16). Drug resistance within the viral population has proven to be a major problem for the long-term treatment with a mono-therapy. It was long thought that after an initial burst of viral replication in the first few weeks post-infection, a period of low-level viremia was maintained for many years. It was assumed that these years of clinical latency were synonymous with low viral burden. However, this has recently been shown to be incorrect, as high-viral burdens have been detected in the lymphoid tissues of HIV-infected individuals at various stages of disease (17). In addition, HIV is now known to be actively replicating even during the asymptomatic phase of the disease (17, 18).

Two recent reports have shown some remarkable insight on the dynamics of the on-going struggle between the virus and the immune system (19, 20). They demonstrated that patients initiating chemotherapy with one of a number of anti-viral drugs under go a rapid reduction in circulating virus within a few days. There is a concomitant rise in $CD4^+$ T-cell lymphocytes. However, drug-resistant mutants can be detected even within 7 days. Interestingly, these studies showed that there is a tremendous turnover of virus, between 10^8 and 10^9 virions being cleared every day (approximately 30% of the estimated total). Therefore there must be a comparable production of virus to maintain the levels observed during disease progression.

In view of this new understanding of the immunopathology of the disease, it is likely that mono-therapy cannot succeed. Combination therapy is the best alternative, aimed not only at the virus itself, but also at bolstering the ability of the immune system to respond more effectively and for longer period of time.

Cytokine Involvement - It has been postulated for a number of years, that cytokines are intimately involved in the progression of HIV disease and that the associated immune dysregulation is due to a switch in cytokine profile (21, 22). Some have gone as far as to suggest that AIDS is in fact a cytokine-based disease (23). Cytokines are cellular, peptide hormones involved in promoting an inflammatory response and maintaining homeostatic function of the immune response (24). HIV infection stimulates cytokine production and several of these induced cytokines have been shown to impact expression of HIV in a number of different infected cell types (25).

The cytokine tumor necrosis factor-alpha (TNF-α) is probably the key mediator of inflammation and has been linked to the sequelae of many infectious and immune-related diseases and disorders (26, 27). TNF-α has been shown to induce HIV expression in chronically-infected promonocytic and T-cell lines (28, 29). This inductive effect has been correlated with the activation and increased nuclear binding of the cellular transcriptional activator NFκB (30). Various research groups have demonstrated that TNFα regulates pro-viral activation in an autocrine manner independent of cell type (19, 31, 32). Hence it is possible that TNFα plays a central role in the activation of latent HIV with a consequent exacerbation of the disease.

INHIBITION OF TNFα BY THALIDOMIDE

Thalidomide remains the treatment of choice worldwide for erythema nodosum leprosum (ENL), an acute inflammatory reaction occuring in lepromatous leprosy patients, (33). In addition to the severe systemic symptoms (fever, cutaneous lesions, weight loss, etc.) there is also significantly elevated serum levels of the primary inflammatory cytokines i.e. TNFα and interleukin-1ß(IL-1ß) (34). The rapid and dramatic relief of symptoms obtained with thalidomide is coupled with a concomitant drop in circulating serum levels of TNFα. This prompted an investigation of whether the drug could directly influence the cells primarily responsible for the synthesis and release of the cytokine, i.e., the mononuclear phagocytes (35). Thalidomide was shown to selectively inhibit the production of TNFα by human monocytes when stimulated with an agonist such as lipopolysaccharide (LPS) (36). The inhibition of TNFα synthesis in this system is incomplete (60% - 80%), which may be beneficial as TNFα is required for normal immune response. Other inflammatory cytokines such as IL-1ß, IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF) were essentially unaffected (36). Monocytes taken from ENL patients make elevated levels of TNFα in response to an agonist *in vitro* (37). This state of cytokine up-regulation is not observed with monocytes taken from ENL patients undergoing treatment with thalidomide (36).

The mechanism by which thalidomide can down-regulate the production of TNFα by monocytes, is not fully understood. A number of cellular effects have been noted. For example, a drug dependent enhancement of TNFα mRNA degradation in monocytes (37), a down-regulation of a cell surface marker (CD26) on $CD4^+$ T-cell lymphocytes (38), inhibition of a cellular transcriptional activator NFκB. Despite these observations, a definitive mechanism of action(s) has yet to be determined.

Cells latently infected with HIV-1 can be activated and viral replication initiated when TNFα is present as described earlier. Therefore, if thalidomide can effectively down-regulate the production of TNFα, it may reduce or prevent viral expression in such cells. This anti-viral effect has been demonstrated in both latently infected cell lines and in peripheral blood monocytes taken from

infected patients (39). Dose-dependent inhibition as measured by viral reverse transcriptase activity or by viral P24 antigen assays was observed (39). These *in vitro* results have prompted the investigation of the potential clinical use of thalidomide in HIV disease. Interestingly many immunological and clinical manifestations observed in patients infected with HIV are also present in a variety of systemic autoimmune diseases e.g. lupus erythematosus, Behçets syndrome, rheumatoid arthritis, graft-vs.-host disease, etc. (40). Many of these systemic autoimmune diseases respond to thalidomide treatment.

THALIDOMIDE USE IN HIV/AIDS

Clinical Status - Before the possible "anti-viral" properties of thalidomide were apparent, the drug had already proved an effective weapon against one of the more debilitating sequelae of HIV/AIDS, recurrent aphthous ulceration. These lesions cause severe pain and dysphagia and are often unresponsive to conventional treatments e.g., antibiotics, steroids. Even though recurrent aphthous stomatitis is the most common disease effecting the oral mucosa, there is thought to be an increased incidence in HIV infected individuals (41, 42). Thalidomide has been a remarkably effective therapy in the most recalcitrant cases of aphthous ulceration in patients who are HIV-1 seropositive (42-48). Long standing remission has been seen in approximately half of such patients with others experiencing temporary remission lasting several months. A placebo-controlled blinded study evaluating the use of thalidomide in the treatment of aphthous ulcers in HIV/AIDS patients is currently underway in the U.S. sponsored by the National Institute of Allergy and Infectious Disease (NIAID).

Another debilitating complication most commonly observed with HIV patients with advanced disease is cachexia or wasting syndrome. This is severe weight loss (> 10% ideal body weight) that cannot be attributed to any etiological agent. Cachexia is also associated with a variety of chronic diseases e.g. tuberculosis and also with advanced cancer. The cause of this wasting syndrome has also been linked to elevated levels of serum TNFα (27, 49-53).

Two pilot studies have evaluated the treatment of cachexia in HIV/AIDS patients with thalidomide (54, 55). A randomized, placebo-controlled double blind study was conducted in Thailand (54). Half of the 32 patients received a single dose of thalidomide (300 mg) for 21 days. The treatment group averaged 4.5% weight gain over the three weeks. The placebo group exhibited weight gain of around 1%. A similar controlled study was carried out in Mexico (55). The treatment period was 12 weeks with a dose of 100 mg four times a day. Eighteen patients completed the protocol (9 placebo and 9 thalidomide). Stabilization or weight gain was observed in 8 of 9 patients receiving thalidomide as opposed to 2 of 9 patients from the placebo group. The patients $CD4^+$ T-cell counts were not effected in either study. There was

no evidence to suggest that the drug had any impact on viral burden during the treatment period. No major adverse reactions were seen in either study. Somnolence was observed in the treatment groups. Erythematous macular rash was observed in a number of patients and has also been seen in HIV patients being treated for aphthous ulceration. This kind of hypersensitivity reaction is not uncommon in HIV patients receiving chemotherapy. When withdrawn from drug, the rash resolved within a few days. Peripheral neuropathy has been observed in patients receiving chronic administration of the drug (5).

Based on these promising pilot studies, two clinical trials are currently in progress in the U.S. assessing the use of thalidomide in HIV/AIDS and associated complications. The first is a corporate-sponsored, control study evaluating the efficacy of the drug in controlling wasting syndrome associated with HIV/AIDS (56). If the drug effectively reverses the on-going weight loss, the nature of any increased weight will be determined i.e. muscle, fat, etc. The second clinical study is sponsored by NIAID, a division of the National Institutes of Health. The study will evaluate the safety, pharmacokinetics and the effect of thalidomide on the progression of HIV/AIDS disease (57).

Clinical Future - The clinical use for thalidomide in combatting HIV/AIDS and associated complications has potential on three different levels.

Level One - The ability of the drug to impact on the virus itself, i.e. anti-viral activity. As discussed, the drug has exhibited anti-viral activity indirectly by inhibiting TNFα production. The pathogeny of HIV infection has been postulated to really be an abnormal host immunological response and not related to the direct cytotoxic effect of the virus (58). If this hypothesis is correct or partially correct, then there may be a role for thalidomide controlling the disease as in other auto-immune diseases.

There appears to be a role for thalidomide as a co-therapy for the disease, either in combination with current or developing anti-viral compounds or in combination with other immune system stimulants or restoratives. A recent study used interleukin-2 (IL-2) as an immunomodulatory agent to restore immune function in HIV-infected individuals (59). The results indicate that significant improvement as measured by increased $CD4^+$ lymphocyte counts could be realized in patients with $CD4^+$ counts originally > $200/mm^3$. These exciting results were tempered only by the presence of toxic side effects associated with the IL-2 infusions. As a number of these adverse reactions look similar to those observed in ENL and could be TNFα related, there may be a role for thalidomide as an IL-2 co-therapy.

Level Two - There are a number of immunological based symptoms and complications that can arise subsequent to HIV infection. Two of the most prominent have already been discussed: aphthous ulceration and wasting syndrome. Any contribution the drug can make to return the immune system into balance without causing general immunosuppression will reduce the

potential for these high morbidity sequelae.

Level Three - As the degree of immunodeficiency increases as a result of HIV infection, the frequency of additional opportunistic infections increases. These infections can be triggered by a variety of etiological agents, viruses, bacteria, fungi and protozoans. A poor immune response means these infections can become increasingly difficult to treat and can lead to a chronic, persistent condition. Acute mycobacterial infections e.g. tuberculosis (TB), *Mycobacterium avium* complex (MAC) often result in chronic weight loss with attendant fever and malaise. If an AIDS patient is already suffering from wasting syndrome and contracts such an opportunistic infection, the cumulative effect can be devastating and ultimately life-threatening. Thalidomide has been shown to be effective in reversing weight loss associated with tuberculosis, both in HIV-1 sero negative and positive patients (60). Therefore, there is good reason to believe the drug may have significant clinical benefit to a wider group of AIDS patients with concomitant mycobacterial infections. Controlled clinical trials will be initiated this year to establish the efficacy of the drug in such situations.

Gastrointestinal infections caused by various protozoa, e.g. *Microsporidium, Cryptosporidium*, can lead to chronic diarrhea accompanied by abdominal pain and anorexia (61). There is virtually no effective treatment for these conditions which become chronic and patients can undergo severe weight loss. Symptoms are eventually often managed with high dose opiates. Elevated levels of TNFα have been detected in stools of patients infected with *Microsporidium* (62) and have been shown to severely depress gut adsorption (63). Hence thalidomide may be a useful agent in treating the symptoms of these conditions.

CODA

The clinical future for thalidomide in the treatment of HIV/AIDS and some of the more devastating complications looks promising from a scientific and clinical perspective. However, there are political and ethical questions that permeate the clinical development of this drug. Recent guidelines from both the NIH (64) and the FDA (65) have recommended the inclusion of women of child-bearing potential in clinical trials. This had lead to some heated debate within Institutional Review Boards (IRB) reviewing protocols involving thalidomide (66). Inclusion of women of child-bearing potential onto a study using thalidomide currently requires pregnancy testing combined with the use of one barrier and one hormonal method of contraception simultaneously. This situation raises many issues e.g. the potential risk of fetal malformation vs. the inherent right of women to have access to investigational therapy. Is institutional liability a valid reason to exacerbate an already prejudiced attitude concerning women with AIDS? Is there a comparable concern over existing approved and non-approved drugs that women with AIDS take on a routine basis? Perhaps thalidomide can catalyse more healthy discussion of these topics seminal to all present and future drug development.

There is more chemical and clinical information available for this drug than for most other investigational drug compounds or even approved drugs. It should be possible in an age where the American healthcare system can safely provide Accutane® (isotretinoin a known, potent teratogen) to treat acne in predominately teenage girls that appropriate measures can be taken for the safe administration of thalidomide for life-threatening complications associated with AIDS.

When the political and ethical dust settles and clinical trials are completed, it may be possible that the drug responsible for one of the greatest man-made medical tragedies of the century will gain approval for use against the greatest medical catastrophe of the century.

References

1. F.O. Kelsey, Teratol., 38, 221 (1988).
2. J. Sheskin, Clin. Pharmacol. Ther., 6, 303 (1965).
3. H.P. Koch, Prog. Med. Chem., 22, 165 (1985).
4. P.F. D'Arcy and J.P. Griffen, Adverse Drug React. Toxicol. Rev., 13, 65 (1994).
5. V. Gunzler, Drug Safety, 7, 116 (1992).
6. T.L. Chen, G.B. Vogelsang, B.G. Petty, R.B. Brundrett, D.A. Noe, G.W. Santos and O.M. Colvin, Drug Met. Dis., 17, 402 (1989).
7. T. Eriksson, S.B. Jörkman, B. Roth, Å. Fyge and P. Höglund, Chirality, 7, 44 (1995).
8. E.P. Sampaio, E.N. Sarno, R. Galilly, Z.A. Cohn and G. Kaplan, J. Exp. Med., 173, 699 (1991).
9. R.J. D'Amato, M.S. Loughnan, E. Flynn and J. Folkman, Proc. Nat. Acad. Sci. USA, 91, 4082 (1991).
10. S. Fabro, R.L. Smith and R.T. Williams, Nature, 215, 296 (1967).
11. G. Blaschke, H.P. Kraft, K. Fickentscher and F. Kohler, Arzniem-Forsch., 29, 1640 (1979).
12. B. Knoche and G. Blaschke, J. Chromatogr., 666, 235 (1994).
13. M.A. Fischl, D.D. Richman, M.H. Grieco, M.S. Gottlieb, P.A. Volberding, O.L. Laskin, J.M. Leedom, J.E. Groopman, D. Mildvan, R.T. Schooley, G.G. Jackson, D.T. Durack, D. King, and the AZT Collaborative Working Group, N. Engl. J. Med., 317, 185 (1987).
14. D.D. Richman, Antimicrob. Agents Chemother., 37, 1207 (1993).
15. M.I. Johnston and D.F. Hoth, Science, 260, 1286 (1993).
16. C.B. Pettinelli and S.M. Schnittman, Infec. Agents Dis., 2, 291 (1994).
17. G. Pantaleo, C. Graziosi, J.F. Demarest, L. Butini, M. Montroni, C.H. Fox, J.M. Orenstein, D.P. Kotler and A.S. Fauci, Nature, 362, 355 (1993).
18. R.I. Connor, H. Mohri, Y. Cao and D.D. Ho, J. Virol., 67, 1772 (1993).
19. X. Wei, S.K. Ghosh, M.E. Taylor, V.A. Johnson, E.A. Emini, P. Deutsch, J.D. Lifson, S. Bonhoeffer, M.A. Nowak, B.H. Hahn, M.S. Saag and G.M. Shaw, Nature, 373, 117 (1995).
20. D.D. Ho, A.U. Neumann, A.S. Perelson, W. Chen, J.M. Leonard and M. Markowitz, Nature, 373, 123 (1995).
21. M. Clerici and G.M. Shearer, Immunol. Today, 14, 107 (1993).
22. M. Clerici and G.M. Shearer, Immunol. Today, 15, 578 (1994).
23. T. Matsuyama, N. Kobayashi and N. Yamamoto, AIDS, 5, 1405 (1991).
24. M.J. Clemens in "Cytokines," Bios. Scientific Publishers, Oxford, UK, 1991.
25. A.S. Fauci, Science, 262, 1011 (1993).
26. K.J. Tracey and A. Cerami, Ann. Rev. Cell Biol., 9, 317 (1993).
27. K.J. Tracey and A. Cerami, Ann. Rev. Med., 45, 491 (1994).
28. T.M. Folks, K.A. Clouse, J.S. Justement, A. Rabson, E. Duh, J.H. Kehrl and A.S. Fauci, Proc. Nat. Acad. Sci. USA, 86, 2365 (1989).
29. G. Poli, A. Kinter, J.S. Justement, J.H. Kehrl, P. Bressler, S. Stanley and A.S. Fauci, Proc. Nat. Acad. Sci. USA, 87, 782 (1990).

30. E.J. Duh, N.J. Maury, T.M. Folks, A.S. Fauci and A.B. Rabson, Proc. Nat. Acad. Sci. USA, 86, 5974 (1989).
31. A. Vyakarnam, J. McKeating, A. Meager and P.C. Beverly, AIDS, 4, 21 (1990).
32. K. Kitano, C.I. Rivas, G.C. Baldwin, J.C. Vera and D.W. Golde, Blood, 82, 2742 (1993).
33. L.K. Bhutani, Lancet, 345, 697 (1995).
34. E.N. Sarno, G.E. Grau, L.M.M. Viera and J.A. Nery, Clin. Exp. Immunol., 84, 103 (1992).
35. E.P. Sampaio, A.L. Moreira, E.N. Sarno, A.M. Malta and G. Kaplan, J. Exp. Med., 175, 1729 (1992).
36. E.P. Sampaio, G. Kaplan, A. Miranda, J.A.C. Nery, C.P. Miguel, S.M. Viana and E.N. Sarno, J. Infec. Dis., 168, 1675 (1993).
37. A.L. Moriera, E.P. Sampaio, A. Zmuidzinas, P. Frindt, K.A. Smith and G. Kaplan, J. Exp. Med., 177, 1675 (1993).
38. R. Neubert, H. Helge and D. Neubert, Life Sci., 56, 407 (1995).
39. S. Makonkawkeyoon, R.N.R. Limson-Pobre, A.L. Moreira, V. Schauf and G. Kaplan, Proc. Nat. Acad. Sci. USA, 90, 5974 (1993).
40. V. Günzler, Med. Hypot., 30, 105 (1989).
41. G.M. Connolly, D. Hawkins, J.N. Harcourt-Webster, P.A. Parsons, O.A.N. Husain and D.B.G. Gazzard, Gut, 30, 1033 (1989).
42. D.P. Nicolau and T.E. West, Drug Intel. Clin. Pharm., 24, 1054 (1990).
43. M. Youle, J. Clarbour, C. Farthing, M. Connolly, D. Hawkins, R. Staughton and B. Gazzard, Brit. Med. J., 298, 432 (1989).
44. J. Ryan, J. Colman, J. Pedersen and E. Benson, N. Engl. J. Med., 327, 208 (1992).
45. B. Radeff, R. Kuffer and J. Samson, J. Am. Acad. Dermatol., 23, 523 (1990).
46. G. Ghigliotti, T. Repetto, A. Farris, M.T. Roy and R. DeMarchi, J. Am. Acad. Dermatol., 28, 271 (1993).
47. I. Bodokh, J.Pl. Lacour, L. Rainero, J. Castanet, J.F. Michiels and J.P. Ortonne, La Presse Med., 22, 1233 (1993).
48. D.L. Paterson, P.R. Georghiou, A.M. Allworth and R.J. Kemp, Clin. Infect. Dis., 20, 250 (1995).
49. B. Beutler and A. Cerami, Adv. Immunol., 42, 213 (1988).
50. A. Cerami, Blood Purif., 11, 108 (1993).
51. B.M. Speigelman and G.S. Hotamisligil, Cell, 73, 625 (1993).
52. N.J. Espat, E.M. Copeland and L.L. Moldawer, Sur. Oncol., 3, 255 (1994).
53. M.A. O'Connell, D.G. Weir and D. Kelleher, Eur. J. Gastroen. Hepat., 6, 821 (1994).
54. J.D. Klausner, S. Makonkawkeyoon, P. Akarasewi, W. Kasinrerit, K. Nakata and G. Kaplan (ABS) 10th International Conference on AIDS, Yokohama, 7-12 Aug, 1994.
55. G. Reyes-Teran, J.G. Sierra-Madero, V. Martinez del Cerro, T. Munoz-Trejo, H. Arroyo-Figueroa, A. Pasquetti, J.J. Calva and G.M. Ruiz-Palacios, (ABS) 10th International Conference on AIDS, Yokohama, 7-12 Aug., 1994.
56. Scrip, 1915, 27 (1994).
57. Scrip, 2010, 24 (1995).
58. J.C. Barbosa Vosgerau, Panmin. Med., 36, 1 (1994).
59. J.A. Kovacs, M. Baseler, R.J. Dewar, S. Vogel, R.T. Davey, J. Falloon, M.A. Polis, R.E. Walker, R. Stevens, N.P. Salzman, J.A. Metcalf, H. Masur and H.C. Lane, N. Engl. J. Med., 332, 567 (1995).
60. J.M. Tramontana, U. Utaipat, A. Molloy, P. Akarasewi, M. Burroughs, S. Makonkawkeyoon, B. Johnson, J.D. Klausner, W. Rom and G. Kaplan, Mol. Med. (in press).
61. M. Wittner, H.B. Tanowitz, and L.M. Weiss, Infect. Dis. Clin. North Am. (US), 7, 569 (1993).
62. D. Sharpstone, N. Rashid, G. Arthur, A. Rowbottom and D.B.G. Gazzard, Gut, 35, 40 (1994).
63. G. Singh, K.L. Chaudry, M.H. Morrison and I.H. Chaudry, Circ. Shock (US), 39, 279 (1993).
64. Fed. Reg., 59, 1994 (1994).
65. Fed. Reg., 58, 39406 (1993).
66. T. Killip and N. Harlow, J. Am. Med. Wom. Assoc. (US), 49, 122 (1994).

Chapter 33. Educating Medicinal Chemists

C. Robin Ganellin, Lester A. Mitscher,* John G. Topliss**
Department of Chemistry, Christopher Ingold Laboratories, University College of London, 20 Gordon St., London, WC1H OAJ, England.
*Department of Medicinal Chemistry, Kansas University, Lawrence, KS 66045, USA.
**Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, 428 Church St., Ann Arbor, MI 48109-1065

Introduction - The pharmaceutical industry is the major employer of organic chemists engaged in research and development of new chemical entities for therapeutic purposes. Many prospective chemist employees are recruited to become industrial medicinal chemists with scant prior exposure to the field itself or to biology. These new employees are expected to fit into a multidisciplinary team of scientists searching for novel molecules with the intention to produce new medicines and to learn most of their craft on the job. When it comes to hiring such chemists the pharmaceutical employer generally seeks out the brightest and most dynamic synthetic organic chemists, most often with a Ph.D. and postdoctoral experience in the laboratory of well known and highly regarded professors of organic chemistry.

The newly appointed chemists have to discover rapidly how to synthesize new molecules suitable for focused biological testing. This usually makes full demands on their synthetic skills and keeps them busy for many months, but is ultimately less than totally satisfying. A fully rewarding intellectual experience requires an appreciation of the reasons for making the compounds. They must learn to interface with diverse biological scientists, for example, to converse knowingly with biochemists, pharmacologists, toxicologists molecular biologists, and formulation scientists in order to be actively involved in the drug design. They will increasingly want to decide what compounds to make, as well as figuring out how to make them. Explosive rates of increase in knowledge in collateral fields such as pathophysiology, cell biology, and genetics must be assimilated at the same time.

Soon the novice medicinal chemist will also come up against the mystery of trying to relate chemical structure to biological activity in the face of insufficient data. Colleagues will mention the importance of lipophilicity, hydrophobic interactions, quantitative structure-activity relationships (QSAR), molecular modeling, electrostatic energy potentials, partial agonists, etc. These are often unfamiliar words and new concepts that will be difficult to acquire and which take time to comprehend and use appropriately. The advent of combinatorial chemistry techniques would seem to place an even higher premium on dealing with student needs for suitable biological background in order to make optimum use of this technique. Very few organic chemists will have taken courses in drug design during their university training. Some may have taken courses in natural product chemistry or bioorganic chemistry but these topics will be of comparatively little help in attacking structure-activity analysis in a pro-active manner. The extensive mentoring which was a feature of previous days is eroded by the present wave of down sizing and enforced early retirements of experienced practitioners.

Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved

One may ask whether such chemists have had adequate University training and whether they should have received much more exposure to medicinal chemistry concepts while at University. In the United States Departments of Medicinal Chemistry not only train pharmacists but also grant graduate degrees in Medicinal Chemistry. Pre- and postdoctoral training in such a setting is one possible solution to the problem posed. Many such departments, however, provide a lesser emphasis on mastering synthetic methods than departments of organic chemistry which in their turn provide comparatively less exposure to modern biological concepts.

This question of the most appropriate educational site and content is international in scope. In Germany and the UK the pharmaceutical companies generally hire organic chemists rather than medicinal chemists who have been trained in Schools of Pharmacy. In Italy every university has a pharmacy faculty and students can opt for a research doctorate in "chemistry and pharmaceutical technologies" (the Italian "CFT") but still companies appear to prefer to take on organic chemists. In Japan the situation is somewhat different since the majority of medicinal chemists in the pharmaceutical industry have graduated from faculties of pharmaceutical science where they have also specialized in organic chemistry. It is common for pharmaceutical companies in Japan to hire medicinal chemists with a Master's degree who have studied courses in pharmaceutical science at university; some have obtained a PhD in addition and quite a number of the chemists take a PhD while working at the company. Thus the situation for the training of medicinal chemists is different in Japan since they have generally studied biologically-based subjects before graduating.

Questionnaire Sent to Companies - This issue of the optimal educational experience for medicinal chemists has been a matter for discussion by the Medicinal Chemistry Section of The International Union of Pure and Applied Chemistry (1). A Working Party has been set up to consider a Medicinal Chemistry Curriculum which addressed these issues (2). It was decided also to seek the viewpoint, from an industrial perspective, of what academic training (if any) should be provided in medicinal chemistry and a questionnaire was devised. This questionnaire was sent out (initially in July 1992 and subsequently again in July 1993) to research directors or directors of medicinal chemistry in major research pharmaceutical companies in various countries. Replies were received from approximately 67 companies, the greatest numbers of which were in Germany, Italy, Japan, UK and the USA. The companies were also invited to photocopy the questionnaire and distribute it to their senior medicinal chemists in research. A total of 103 replies were received and these provided the data which were analyzed.

The respondents vary in industrial medicinal chemical experience with 10-25 years being representative. Their level of responsibility varies from group leader to vice president and the cadre of chemists reporting to them ranges from 2 to 50 or more. All indicate that they either have direct responsibility for new hires or have an influential role in such decisions. Not surprisingly the majority received their formal training in synthetic organic chemistry. The firms represented are predominantly big and multinational. Small firms are more interested in generalists whereas larger firms tend toward specialists. Academics were not polled at this time.

The problem of suitable training touches all of us involved in medicinal chemical research. Because the replies were somewhat startling and provocative, we thought

that it would be of interest to share the results widely within the community of active medicinal chemists. Medicinal chemists, like other chemists, may take up various jobs, but it is clear from the questionnaire replies that the industrial chemists focused their primary attention on the design and synthesis of new compounds for biological testing. No doubt, this was assisted by the preamble of the questionnaire which stated: *Medicinal chemists involved in new drug discovery have to be outstandingly good in synthetic organic chemistry and to understand modern approaches to structure-activity analysis. In addition they need to appreciate important concepts at the interfaces with the many other scientific disciplines involved in the drug design process. In the past, most medicinal chemists were organic chemists who had to acquire the language and concepts needed to become medicinal chemists "on the job". This takes a long time, however, and, as is well known, not all organic chemists make a good transition. It should be possible to greatly accelerate the learning process by introducing appropriate training either whilst chemists are still at University or shortly afterwards. We would value your opinion on this issue."*

Several answers indicated that there was no need for medicinal chemistry to be taught in universities, because the subject could be taught adequately within the company. On the other hand, this was more than counterbalanced by the replies that wanted organic chemists foremost but also preferred them to have an awareness of biologically-based subjects such as biochemistry, pharmacology, physiology and molecular biology.

Results From Analysis of the Answers Received - The questionnaire was comprised of seven main questions plus a request for information about the respondent (position, responsibility, training and involvement in drug discovery). A report analyzing the replies mainly from Germany, Japan and the UK, but also with a few from other countries, has been published (3). In the present chapter the results are tabulated for the USA responses (4) and compared with the totals from Germany, Italy, Japan and UK. The North American responses were generally consistent with those from Europe. The main differences being the willingness of non-USA responders to place a greater emphasis on preacquired biological skills.

Question 1 - *When recruiting a new medicinal chemist, what do you look for in terms of knowledge, expertise, qualification?*

A large majority of answers indicated (Table 1) that they were seeking chemists with advanced knowledge in organic chemistry (over 80%) and expertise in organic synthesis. Indeed, over half suggested that they required a PhD with postdoctoral experience in synthetic organic chemistry. Among the additional knowledge and qualification desired, biochemistry figured quite highly, and pharmacology and molecular biology had some mention. Surprisingly few answers mentioned molecular modeling or experience in computing.

Question 2 - *Do you prefer to employ A: specialists in medicinal chemistry who have acquired expertise in synthetic organic chemistry, or B: organic chemists with additional education in medicinal chemistry?*

A very high percentage (>90%) gave their answer as "B". Many of the answers

indicated very strongly that the basic education must be in synthetic organic chemistry since medicinal chemistry can be acquired later whereas it is extremely difficult to do the reverse, i.e. to learn a high level of expertise in organic synthesis after leaving University training.

Table 1 - Answers to Question 1

The subjects mentioned have been grouped together under the most appropriate subdiscipline heading in the first column. The second column shows the number of US replies out of a possible 33, and the third column indicates this as a percentage. Since many respondents mention more than one subject, the combined percentages are greater than 100. Columns 4 and 5 show the numbers of replies (out of 60) received from outside the US and the corresponding percentages.

	US replies		Non-US replies	
Knowledge				
Organic Chemistry	29	87%	46	77%
Biochemistry	9	27%	10	17%
Physical Organic Chemistry	5	15%	1	2%
Medicinal Chemistry	4	12%	2	3%
Pharmacology	2	6%	9	15%
Molecular Biology	1	3%	3	5%
Modeling	0		3	5%
Expertise				
Synthetic Organic Chemistry	20	60%	33	55%
Computer Experience	1	3%	6	10%
Pharmacology/Biology	3	9%	2	3%
Qualification				
PhD in Organic Chemistry	2	6%	33	55%
Postdoctoral	18	54%	14	23%

Question 3 - *If you were to employ a synthetic organic chemist who will join your company to become a medicinal chemist, please indicate your priorities for additional education against the listed subject disciplines, and indicate the stage at which each subject should be acquired.*

Biochemistry was not mentioned among the subjects listed (Table 2) but was broken down into *biological chemistry* (which was identified as encompassing enzyme mechanisms, nucleotides, proteins and peptides, carbohydrates, membranes), *drug metabolism* and *pharmacokinetics*. The answers are summarized in Table 2. It is clear that there is quite a diversity of opinion as to priority rating and stage for learning.

Almost all replies identified synthetic methodology (97%) and a mechanistic approach to synthetic chemistry (93%) as having the highest priorities (ratings 4 and 5) for additional education at undergraduate, graduate and postdoctoral stages. High

priority (3-5) was also given to biological chemistry (at all stages), physical organic chemistry and to a general knowledge of drugs and drug action (but the latter should be left to stage d, in industry). Biological subjects such as molecular biology, pharmacology and bioassay had medium to low priorities as did drug metabolism and pharmacokinetics (mainly stage d). Computational modeling and QSAR theories and correlation analysis had medium priorities (rated 3-4 at all postgraduate stages). Specialist topics like analytical chemistry, process development and pharmaceutical technology received a medium to low rating (2-3). Specialist biological subjects like physiology and toxicology had low priorities (rated 1-3) and they should be left to stage d, in industry. Microbiology was rated even lower (1-2).

Table 2 - Answers to Question 3

The number of responses for each subject is shown for the respective priorities and stage for teaching. Returns for the latter add up to more than 100% because many respondents indicated that subjects should be acquired at more than one educational stage. The total number of respondents to this question was 30.

Subjects	Priorities for Additional Education						Stage for Teaching			
	1	2	3	4	5	Total Score[a]	a	b	c	d
Synthetic Methodology	0	0	1	3	26	145	10	27	14	3
Mechanistic Synthesis	0	1	1	13	15	132	12	24	10	3
Theories of Drug Action	2	6	10	9	4	108	4	5	8	20
Biological Chemistry	1	5	7	10	7	107	7	12	11	8
Physical Organic Chemistry	0	2	14	9	5	107	12	24	7	2
QSAR	1	5	12	9	3	98	0	9	9	15
Computational Modeling	1	2	18	7	2	97	0	12	15	12
Pharmacology and Bioassay	4	6	5	11	4	95	1	5	9	19
Metabolism	3	9	8	8	2	87	0	3	6	22
Molecular Biology	4	5	12	4	4	86	6	9	11	14
Pharmacokinetics	4	9	9	5	3	84	0	3	5	22
Prodrugs	7	6	8	7	2	81	0	1	5	25
Process Development	3	12	10	4	1	78	0	6	3	24
Analytical Chemistry	6	7	11	4	1	71	18	9	2	3
Toxicology	9	8	10	2	1	68	2	2	3	23
Physiology	7	11	9	3	0	68	7	5	4	17
Pharmacy	13	8	8	1	0	57	2	1	2	24
Microbiology	13	11	4	2	0	55	8	5	2	17

[a]Total score represents the sums of the number of answers times the priority values. Maximum score is 150. Minimum is 30.

Question 4 - *If additional courses in medicinal chemistry were to be offered as options to chemistry students, what should be their contents?*

This is an open question. The possible courses were not identified in the question but they were mentioned already in question 3. The question also does not differentiate between undergraduate or graduate students. Answers are listed in Table 3.

Subjects cited by a substantial proportion of respondents are molecular modeling (36%), pharmacology or physiology (33%), molecular biology (30%), enzymology (24%), biochemistry or drug metabolism and pharmacokinetics (21%), and drug design (18%). By and large the answers from US and non-US respondents were similar.

Table 3 - Answers to Question 4

Subject	Number of Responses			
	US	%[a]	Non-US	%[b]
Modeling or QSAR	12	36%	21	35%
Pharmacology or Physiology	11	33%	19	32%
Molecular Biology	10	30%	13	22%
Enzymology	8	24%	9	15%
Biochemistry/Drug Metabolism & Pharmacokinetics	7	21%	14	23%
Drug Design	6	18%	2	3%
Case Histories of Drug Discovery	2	6%	7	12%
Drug Action	3	9%	8	13%
Biological Chemistry	3	9%	7	12%
Toxicology	1	3%	3	5%
Microbiology	1	3%	2	3%
Cell Biology	0	0%	5	8%

[a]Percentage out of a possible total of 33. [b]Percentage out of a possible total of 60.

Question 5 - *Should Universities provide specific courses for medicinal chemists already employed in industry? If yes, please list topics in your priority order.*

This is another open question in which the possible subject courses were not identified, they had, of course, already been mentioned in question 3. The question does not identify how the courses should be organized, e.g. for a short period (one or two weeks), full time, or as a series of evening lectures. Answers are listed in Table 4. It is noteworthy, and rather surprising, that 20% of respondents felt that such curricula should not be offered at all! Subjects cited by a substantial proportion of respondents are pharmacology or physiology (39%), QSAR or molecular modeling (30%), molecular biology (30%), biochemistry (30%) and drug design (18%).

Table 4 - Answers to Question 5

Subject	Number of Responses			
	US	%[a]	Non-US	%[b]
Pharmacology or Physiology	13	39%	18	30%
Biochemistry/Drug Metabolism & Pharmacokinetics	10	30%	17	28%
Modeling or QSAR	10	30%	20	33%
Molecular Biology	10	30%	10	17%
Drug Design	6	18%	1	2%
Enzymology	4	12%	3	5%
Toxicology	2	6%	7	12%
Biological Chemistry	2	6%	8	13%
Organic Synthesis	1	3%	5	8%
Should not be offered	6	20%	6	10%

[a]Percentage out of a possible total of 33. [b]Percentage out of a possible total of 60.

Question 6 - *Please list your suggestions for a medicinal chemistry curriculum.*

This was not posed as a question and it was not specified as to when such a curriculum should be taught. It is presumed that the respondents were giving their views as to what subjects in general constitute the knowledge base for medicinal chemistry. Answers are listed in Table 5. No single subject is mentioned by more than 27% of respondents. The most popular subjects were biochemistry or pharmacokinetics and drug metabolism (27%), pharmacology or physiology (24%), synthetic organic chemistry (24%), molecular modeling or QSAR (21%) and drug design (18%).

Table 5 - Answers to Question 6

Subject	Number of Responses			
	US	%[a]	Non-US	%[b]
Biochemistry/Drug Metabolism & Pharmacokinetics	9	27%	9	15%
Pharmacology or Physiology	8	24%	7	12%
Synthetic Organic Chemistry	8	24%	7	12%
Modeling or QSAR	7	21%	6	10%
Drug Design	6	18%	1	2%
Enzymology	5	15%	1	2%
Biological Chemistry	5	15%	3	5%
Molecular Biology	5	15%	3	5%
Toxicology	3	9%	3	5%
Microbiology	2	6%	2	3%

[a]Percentage out of a possible total of 33. [b]Percentage out of a possible total of 60.

Question 7 - *Having regard to the present recruiting situation in your country, what suggestions do you have for the future to better train chemists in medicinal chemistry?*

Various suggestions were made in answer to this question and a paraphrased selection is listed below:

- Appoint more medicinal chemistry professors from industry.
- Provide additional courses to chemistry graduate students in topics related to drug design, e.g. biochemistry, biological chemistry, molecular biology, pharmacology, computing skills.
- Provide summer training in a medicinal chemistry laboratory during undergraduate or graduate studies.
- Provide university short courses in aspects of drug design to postdoctoral researchers who are working in industry.
- Improve the capability and desire to work in a team and the ability to identify, prioritize and address commercial issues in addition to science.
- Reverse the trend in which the number of synthetic organic chemistry professors in the USA is declining due to decreased funding and the number of students taking this subject is also falling.

Anecdotal Remarks - Some interesting remarks were written on the forms which illustrated a very wide spectrum of viewpoints, some very forcefully expressed. The view was expressed many times that a strong background in synthetic organic chemistry is essential for success. It is a skill that cannot be learned on the job. At one extreme was the statement "we do not ask for additional education in medicinal chemistry since we find that this dilutes the understanding of basic principles in chemistry." Furthermore, several answers indicated that medicinal chemistry and drug research is better taught in industry than in academic institutions. In contrast, one respondent stated: "I want to emphasize that the role of a medicinal chemist is to solve biological problems with an exact science in an interdisciplinary environment. The chemists must not look at themselves in isolation. If they do, they will end up being told what to do! This is one of the underlying problems as to why medicinal chemists are getting short shrift in many companies now. We collectively are to blame for this!" Another indicated: "most of our Ph. D. chemists have strong organic backgrounds and look for similar chemists who can learn medicinal chemistry. It would be preferable to have a better balance." It would be very difficult to reconcile such divergent views!

With regard to teaching of chemists in schools of medicinal chemistry, it was made clear that the schools need to become or remain firmly focused on synthetic chemistry. It was suggested that their students too often have done repetitive synthesis using a limited number of reactions and that there should be more emphasis on acquiring a better background which includes an understanding of molecular reactivity. It was also felt that the research projects themselves sometimes reflect poorly on the candidates. Frequently synthesis of multiple simple derivatives of a single compound is described as is the setting up and running of a simple enzyme assay (normally a technician's job in industry). A stronger showing would be made by describing a diversity of chemistry reactions along with a demonstrated knowledge of drug design.

Another respondent indicated that a general area which needs improvement is the relationship between professors of medicinal chemistry and industry. It was felt that more industry support (including financial support) would help to improve the quality of students available as well as the quality of the academic staff itself.

Conclusions - Those medicinal chemistry departments which place a strong emphasis on synthetic organic chemistry and place their graduates in suitable synthesis-orientated postdoctoral situations in organic chemistry departments are preparing their students as well as possible to compete for the discovery research jobs involving synthesis available in the pharmaceutical industry. Those having a different emphasis are preparing their students by and large for other types of careers. Those chemistry departments preparing students for industrial careers in drug discovery would do well to maintain their synthetic focus and to introduce their students to modern drug design concepts.

In the research based pharmaceutical companies most medicinal chemists are employed in the design and synthesis of new compounds for biological testing as part of the new drug discovery process. It was obvious that this was the basis for the replies received. The overwhelming response was clear. The companies wish to recruit first-rate synthetic organic chemists. Since synthetic organic chemistry is a more basic science it must be made secure; medicinal chemistry is viewed as being capable of being grafted on but the points as issue are when and how. There are many diverse opinions. One extreme view expressed several times was that medicinal chemistry can be learned by experience and that there is no need to teach it in University. If it is taught it must not be at the expense of specialist organic chemistry. Most responses were less extreme. Senior chemists responsible for hiring new medicinal chemists looked favorably on additional courses, such as biochemistry, biological chemistry, pharmacology, physiology, molecular biology, QSAR and molecular modeling.

One issue that was posed by a substantial number of the respondents was whether one can "teach medicinal chemistry" at University or has it to be learned on the job since drug design is an applied subject. This seems to be a misunderstanding by some respondents about the aim of the questionnaire. It takes many years to gain sufficient experience "on the job" for drug design. One answer indicated 15 years! The point of teaching some ideas and methods is not to replace experience but to enhance it. The purpose of teaching is to shorten the time it takes to achieve perceptive understanding and to get the scientist to the creative cutting edge much faster. Nowadays, the molecular basis for drug design is expanding so rapidly that scientists cannot afford the luxury of taking ten or more years for "on the job" training. The point is surely not whether one can teach how to design drugs but whether one can teach some principles and language to be used as tools and where this might be done most effectively. What these tools are will provide the basis for a curriculum in medicinal chemistry.

The practice of medicinal chemistry and its role in drug research continuously changes due to changes in technology and the scientific underpinnings of the field. Forty years ago the industry was dominated by organic chemists. Medicinal chemists were rather rare. Most leads were generated by screening. Since then we have had a period in which a more rational approach has emerged based upon a knowledge of enzymes and receptors as targets. In this period the dialogue between the chemist

and biologist became very important and chemists had to learn much more about the biological targets and how to match the chemistry of drugs to bind to these targets.

We have now entered another phase where technology has provided the opportunity for automated screening against a range of biological targets using radioligand binding or enzyme assays, in addition to screens against microorganisms. Screening 5000 compounds per week to generate a new lead is now commonplace. When a new lead is generated one requires outstanding organic chemistry to follow it up as rapidly as possible by synthesizing analogues. Surely, one has also to select the target molecules for synthesis but the planning requires a brief time compared with the time spent on actual molecule synthesis. Perhaps this underlies the strong bias towards organic chemists shown in these replies. Thus the team will probably include a few experts in nmr spectroscopy, crystallography, chemical computation, a few trained medicinal chemists to relate biology to structure-activity analysis and to solve absorption, distribution, metabolism and excretion problems, but the majority of the team will be the many organic chemists involved in the synthesis.

Regardless of whether the attitudes uncovered by this questionnaire are gratifying or discouraging to the reader, they reflect a pervasive opinion rarely discussed openly and must be dealt with thoughtfully by all those charged with the responsibility of training young chemists for productive careers in industrial medicinal chemistry.

Short Course in Medicinal Chemistry for Industrial New Hires - Brief residential courses in medicinal chemistry for newly hired employees have sprung up to assist with many of the problems discussed in this chapter and these have proven to be very popular. Their general oversubscription clearly indicates that they address a generally felt need. The question of the optimum content of such courses is timely but too extensive for treatment in this space so will be discussed in a separate article.

References and Notes

1. Membership of the Committee is Professor J. G. Topliss (President), Dr. N. Koga (Vice-President), Professor W. D. Busse (Secretary), Professor C. G. Wermuth (Past President), Professor C. R. Ganellin and Professor L. A. Mitscher.

2. The members of the Working Party are Professor C. R. Ganellin (Chairman), Professor W. D. Busse (Bayer, Wuppertal, Germany), Dr. P. Lindberg (Astra, Hassle, Molndal, Sweden), Professor L. A. Mitscher (Kansas University, USA), Professor G. Tarzia (University of Urbino, Italy), Professor C. G. Wermuth (Universite Louis Pasteur, Strasbourg, France) and Dr. R. Ziegler (Sandoz, Basel, Switzerland).

3. W. D. Busse and C. R. Ganellin, Views from industry on the medicinal chemistry curriculum: answers to a questionnaire. In "Trends in Drug Research," V. Claassen, Ed., Pharmacochemistry Library, *20*, Elsevier, Amsterdam, 1993, p 305-315.

4. Letters with the questionnaire were sent to US Companies by Professors L. A. Mitscher and J. G. Topliss.

Chapter 34. Research Investments in a Managed Care, Cost-Containment Oligopsony

W. Leigh Thompson
retired Chief Scientific Officer
Eli Lilly and Company
Chairman and CEO
Profound Quality Resources
54 King Street
Charleston, South Carolina 29401-2731

Prior Success — The pharmaceutical research industry thrived as an oligopoly. Barriers to entry limited competition and a few established firms invented worthwhile cures that were protected by exclusivity. Prices were inelastic—drugs were often so cost–effective that high prices seemed attractive in comparison with the alternatives of death, dsiability, surgery, and hospitalization.

The best measure of the unprecedented success of this system is the prolongation in life expectancy during this century from less than 50 years at the beginning to more than 80 years as this millenium closes. Key to this extraordinary prosperity has been the vaccines, antibiotics, and numerous wonder drugs that now enable keyhole surgery, transplantation, procreation control, and even amelioration of "emotional" disorders. This same capitalistic system has brought us to the dawn of a new era of molecular definition of diseases, medicines made from or found through molecular biology, and gene medicine. We have the potential to cure point mutation diseases in utero, modulate hyperlipidemia, and interrupt the cascade of events leading to cancer.

In the United States these wonders have been achieved while drugs have become ever a smaller fraction of health care spending. When Medicare and Medicaid were enacted in 1965, outpatient prescription pharmaceuticals amounted to almost 10% of health care spending. Today of every dollar spent on health care, less than 4¢ is spent on the prescription drugs taken home and 1¢ is invested in the research that will bring future cures to us and our children.

Research Investments — That research investment by PhRMA members, amounting to almost $15 billion this year, is supplemented by about $3 billion from the smaller biotechnology–focused firms in the United States. Of new molecules marketed in several countries, about half arise from U.S. pharmaceutical research manufacturers and of these less than 8% are discovered in academic or government laboratories.

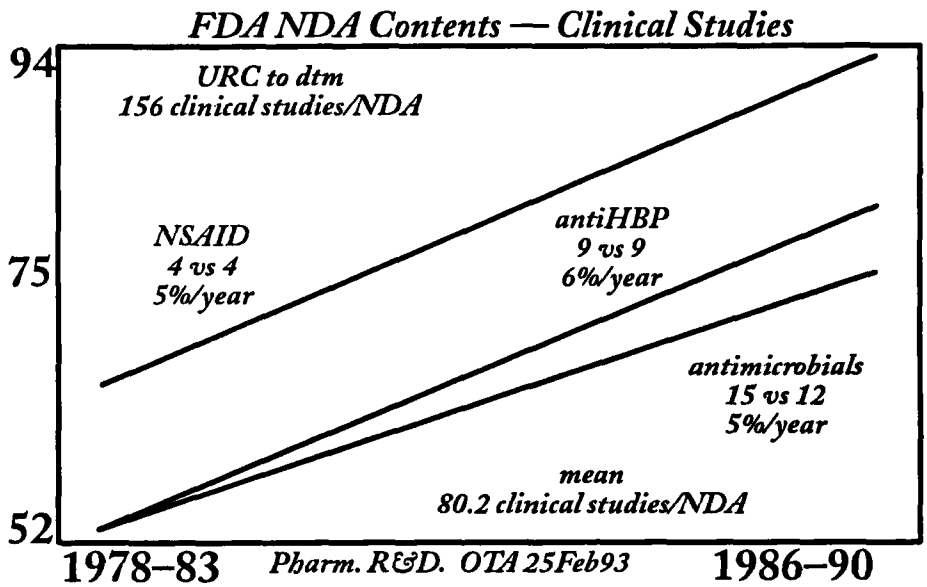
Is the level of health care research appropriate? The federal government invests about 1% of health care spending in research to almost match the investment from private industry. Yet 2% is a very small fraction of such an important aspect of life. The average firm in the U.S. invests more than 3% of its sales in research and Germany and Japan invest even more than the U.S. in non–defense research. But in health care especially we are redefining its scientific bases every day, no one wants last year's technology, and, as Teisberg, Porter, and Brown asserted: "Innovation is the only true long–term solution for high–quality affordable health care."

Is that 1% of health care spending in the United States by PhRMA members wisely invested in new cures? The free enterprise system ensures that waste is minimized as targets are chosen for a marketplace that rewards innovation and cost–effectiveness.

Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

Consider how H2 blockers have minimized ulcer surgery or antipsychotics emptied mental hospitals. The owners of these firms, largely your pension plans, insurance companies, and banks, demand that they focus upon innovations that will be rewarded by serving the greatest true health needs of the largest number of patients. Because the marketplace rewards the innovator who arrives first, the competition among firms ensures proper investments in the infrastructure to ensure speed to market and that a quality product arrives with the appropriate information package.

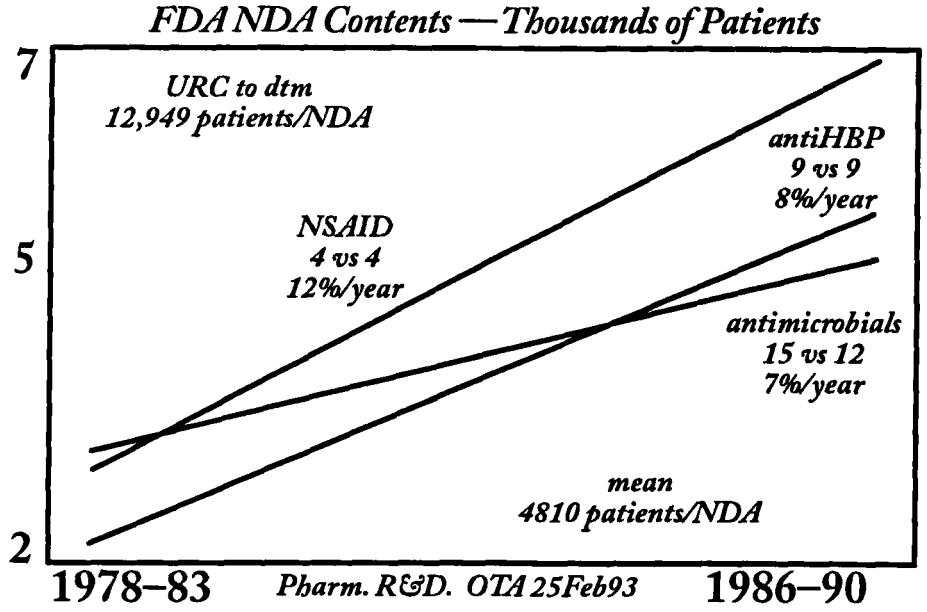
Ominous Prospects — Two factors threaten the viability of this ideal system for adding years to life and life to years. The first is a gradual but progressive effect that has diminished research productivity by about 13% per year for three decades. The U.S. Congress Office of Technology Assessment report on pharmaceutical research, published in 1992, documented this trend. They observed that science inflation exceeds general inflation, but that inflation–adjusted salaries of industry scientists had not increased. In contrast, the cost of the same preclinical studies were rising with a geometric mean of 12% per year. The figures below describe the content of U.S. FDA New



Data from the U.S. FDA on the clinical studies in typical New Drug Applications approved in the years shown as analyzed and published by the U.S. Office of Technology Assessment. Monoexponential rate constants (from two points) are shown as is the very unrealistic extrapolation of these trends to the dawn of the next millenium, the end of the year 2000.

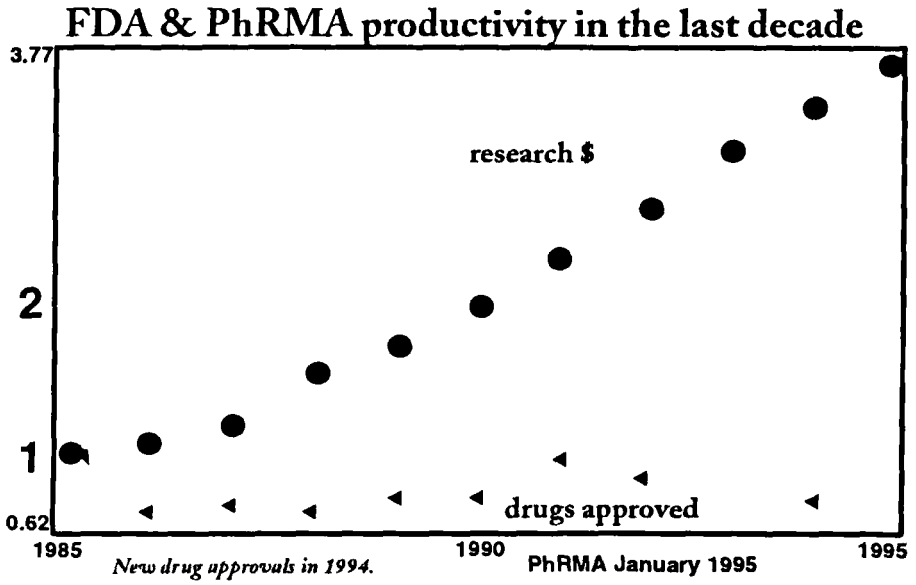
Drug Applications for comparable products approved in two time periods. The number of clinical studies had increased to 80 per NDA. If this trend continued, this number would double by the dawn of the next millenium at the end of the year 2000. Similarly, the number of patients reported in a NDA had reached more than 4,000 and the trend would predict a trebling before the next millenium.

Regulatory Creep — Why have the studies needed for initial marketing of a new cure increased so relentlessly for three decades? I believe it is unrestrained regulatory creep—the well–meaning, inexorable tendency of bureaucrats rewarded only for risk aversion to incrementally demand greater proof for each new approval. If 4000 patients were



Patients studied and reported in typical New Drug Applications at the time of their approval in the years shown. Monoexponential rate constants are shown. Although some NDAs have already contained 13,000 patients, it is unlikely that we could afford any substantial increment in the currently required clinical studies.

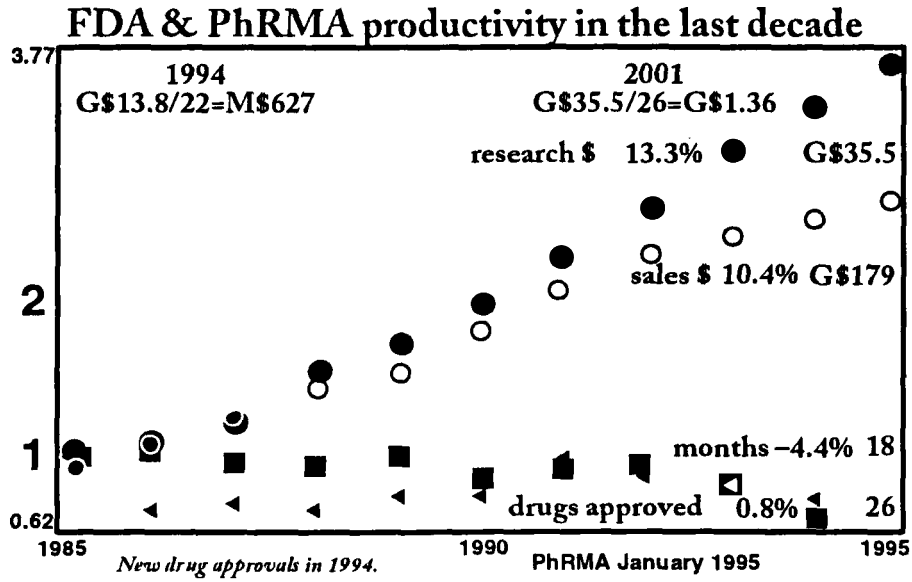
sufficient for the last approval, 4500 is only a small additional number. Now that we have analytical techniques to measure contaminants at ppb, lets replace those ppm standards with more up–to–date ppb ones. Surely if you have systems to prevent defective products from reaching the market we can find systems to ensure the systems that ensure the systems.... The French call this "small steps of tyranny." More patients



with more visits with more variables per visit with more analyses per variable exponentially increment costs as we see in the figure below that plots PhRMA research investments and FDA new molecular drug approvals for the last decade indexed so the 1985 values are set equal to 1. The same trends have existed for three decades as the cost per new approval doubles every five years. In two decades we have added 5 years to the time from invention to first market, in the calculations by Stuart Walker of the Centre for Medicines Research.

How much does a new drug cost? The OTA report updated the figures of Joe DiMasi of the Center for the Study of Drug Development to $359 million, including the cost of capital investments and the cost of research that did not succeed in a new approval. However, these figures reflect drugs first given to humans two decades ago. Correction for the devaluation of our currency does not compensate for the much greater progressive inflation in research costs per approval which in Dr. DiMasi's studies exceeded 10%/year. A more appropriate calculation is to relate the current investments in research for all products to the number of new molecules approved. This avoids a complex calculation of the time value of money and it relates worldwide research investments at all stages to the worldwide sales that must provide the capital to support such research. This ratio for PhRMA members and U.S. FDA approvals in 1994 is $627 million. Stuart Walker calculates worldwide pharmaceutical research investments and worldwide new molecular approvals to be just about twice the U.S. figures, and so this ratio is currently about the same on a worldwide basis. The expanded figure below, from FDA and PhRMA data, shows that research investments have been significantly outstripping growth in sales, that the slow growth in approvals and reduction in approval times of NDAs will not have great impact at the dawn of the next millenium, but the trends might predict an extraordinary cost per approval of $1.36 billion.

There are two ways to consider the implications of regulatory creep on research investments and their recovery. First, PhRMA members spend about 16% of worldwide sales on worldwide research, although the U.S. figures are more than 20%. That means that the average new product approved in 1994 needs to generate sales of about $3.7 billion over its market life. A few will exceed this hurdle, but most will not approach it. If you model the worldwide sale of drugs you will find that with historical durations of exclusivity and research, the break even point was when worldwide peak sales were about the



same as worldwide research investments. Less than two dozen drugs have ever recorded worldwide peak sales of $627 million in today's dollar equivalents.

These trends have been progressing for three decades, and concern was heightening, but the profitability of the pharmaceutical manufacturers in the 1980s made them, the regulators causing the problems, and industry critics complacent. Industry budgeted research as a proportion of current sales rather than projecting what future sales would be needed to recover those investments. If consumers, insurors, and governments continued to increase health care spending at 13% per year, then research investments expanding at the same rate would be safe if they will demand a constant or expanding share of the health care market.

Changing Customers — It was the market changes that drove the owners of pharmaceutical research firms to withdraw one–third or more of their market value in 1990. What were those real and perceived changes? First, health care had become really expensive. When Medicare and Medicaid were begun in 1965 the per capita cost of health care in the U.S. was $200. In 1994 the Department of Commerce measured spending to total $1.06 trillion or $4000 per capita. Because this is also an exponential process, consider the implications for the lifetime cost of health care at 1994 rates: 80 years at $4000/year = $320,000. If we continue to invest one–sixth of earnings in health care, will the average American be able to earn $2 million in a lifetime to afford health care? Aging and a continuing overproduction of physicians drive these costs ever upward, but the White House health plan did not address these critical factors.

Perhaps when America dominated world markets in the 1950s one could be sure of U.S. economic success, but in today's global markets, when excellent software ethers in from Bangalore, can we be so sure of our earning capacity? As our world now puts a premium on bits not atoms, and our K through 12 education trails the developed world, have we dumbed down American competitiveness to the point that high technology firms will move jobs to countries with educated, motivated workers and leave us to accept health at the costs, quality, and earnings of the British health establishment?

Cost or Quality or Both — The short–term evolution of U.S. health care is rapid and as frightening for academic health centers as the pharmaceutical industry. Most workers are now part of managed care plans of one kind or another that actively constrain costs. Medicaid is rapidly becoming managed care. Medicare may follow. Fee–for–saurus medicine will be extinct by 2001. Managed care should compete through explicit demonstration of consistent operations at one point on the cost–quality curve. Then patients can choose to pay more for a plan that guarantees all transplants or to pay less for lower quality. The problem is that quality metrics are illusive and manipulable. The HEDIS 2.0 scale to compare managed care focuses far more on logistics of care or patient happiness than with wellness, capacity, or longevity. Risk adjustment is very difficult. If the academic health center accepts the high risk patients, their raw outcomes data will not be as good and their costs will be higher than the community hospital that refers those high risk patients to them. In the 1960s the academic health centers were an oligopoly where the best health care resided. Today they have trained numerous specialists who can provide care at least as good and at lower cost in the community.

In a decade we will know how to assess quality—meantime will it be cost competition alone? Will your employer sell your health care to the lowest bidder? Do you want your brain surgeon to be the cheapest available? Obviously Americans should be able to spend their own after–tax dollars for whatever health care they prefer, and it was re-

markable that the White House health plan made such discretionary spending a crime and proposed health police to prevent it.

Recovery of pharmaceutical research investments has been changed overnight by the consolidation of decision making in the hands of a few new customers, such as the business managers of managed care organizations, who for populations of patients will dictate use of a cheaper medicine which is not quite as good. This suboptimizing focus on the pharmaceutical line item in the health care budget can lead to very perverse decisions. In Louisiana, New Hampshire, and Germany recent government experiments have reduced spending for pharmaceuticals only to cause far greater spending for physician visits and hospital care. Some pharmaceuticals are truly cost–effective and some patients in a population will respond differently to one drug than another similar pharmaceutical. Even changes in formulation that reduce the frequency of dosing may significantly enhance compliance or minimize peak–to–valley fluctuation in pharmacodynamics. A White House planner or a managed care financial officer can consider what one strategy is best for the entire population, but I hope we don't see tailor shops with one size pants and one size shoes precisely cut for the mean of American sizes.

Prescription for Success — Three interventions for the pharmaceutical industry are urgent. First, regulatory creep must be rolled back to the effective levels of two decades ago. The public health has not been improved by the enormous increases in cost, duration, and risk of pharmaceutical research in those decades. Instead, untold thousands of anonymous corpses line the infinite corridor to approval which is ever lengthened to protect risk averse regulators from the possibility of identifying one patient dead of early approval. As Randy Tobias has said: "When you are down in a hole, first stop digging." We are in a very deep hole of regulatory creep and excessive risk aversion which, as T.S. Eliot said, is: "Not for the good that it will do but that nothing be undone on the margins of the impossible." I urge that scientific decisions about proofs of safety and efficacy, about quality controls and systems of quality assurance, be made by external groups of scientists, validated by an established quality organization such as USP or IOM and composed of cutting edge scientists from academe, government, and industry. FDA should ensure that studies are performed honestly, reported fully, and that agreed standards are applied rigorously, but the scientific basis of those standards should be set by more appropriate scientific fora.

Second, the pharmaceutical research industry must re–engineer vigorously to accomplish more with less. Eighty clinical studies are not required for proofs of safety and efficacy. One worldwide triple–blind dose response study, started just after the initial definition of kinetics and dynamics, may be sufficient. If not one, then certainly a few powerful studies admitting highly heterogeneous patients to predict marketplace drug reactions. Measuring kinetics, efficacy, or safety in a young homogeneous population that, almost by definition, will not be users of the drug provides Gilbert and Sullivan results which, as Thomas Lehrer noted, are: "full of sound and fury and signifying nothing." The re–engineering principle was best expressed by Antoine de Éxupéry who said that: "Elegance is achieved not when there is nothing more to add, but when there is nothing more to take away."

Such elegance may require that the monolithic totipotential traditional companies, that have done everything from discovery through worldwide marketing, adopt a more innovative structure. Research reduces uncertainty. At every milestone one can measure the expected value of the project. Assets are employed to move to the next milestone, eliminating some prospects but enhancing the value of those that remain. The increase in expected value per assets employed is a fundamental measure of research produc-

tivity. Whenever a milestone can be reached at lower cost, faster, or with higher quality with rented assets—rent. Whenever someone offers you more than your expected value of a project—sell. When you can add more value than anyone else—buy. This is the structure of a virtual drug company in which the corporate strategic advantage is the ability to understand with profound knowledge and manage the entire process, not necessarily owning all the assets needed for any stage of research, manufacturing, or marketing.

Third, we must plan with far more elegant strategies extending decades into the future, into small but growing markets, and spanning medical cultures throughout the developed and developing world. The key to success is, as Thomas Watson noted, failing fast. Resources must be withdrawn from projects before they sour, while they still seem good, but realistically not quite as good as alternatives. We must recognize that therapeutic substitution greatly lowers the value of even the most innovative discovery if it is not rapidly first to market and even then its period of exclusivity may be the key to recovering the research investments. Good projects may need to be abandoned just because others will be marketed first or can be manufactured and distributed through protected channels at lower cost. Such considerations have led some firms to vertical integration to move beyond the 5% of health care spent on outpatient prescription drugs but to incorporate more of the other 95% of health care. It is not unlikely that some firms may own health care delivery itself as the pharmaceutical industry as the profound knowledge of care delivery throughout American and other countries, it can diffuse knowledge rapidly, it can influence the behavior of health professionals, it has mastered the informatics of clinical trials on a worldwide basis, and it has recognizes that its information, not molecules, are the real value it generates.

Such decisions are very difficult to make and even more difficult to weave through the diverse research culture. They can be achieved by evolving a credible process that is transparent, in which many scientific experts contribute to estimates of those project they know most about, where managers can calibrate such estimates across the whole portfolio, and where the most senior management decision makers can develop profound knowledge of the intricacies of research, the variable dynamics of the marketplace, and make quantitative resourcing decisions based on sound scientific principles. Decision analysis is not the answer but the language to improve the quality of conversations about important decisions.

The path to recovery of research investments has become far more difficult, but as always innovation and elegance are two of the keys to success in the American free enterprise system. The third, fundamental to our country and our culture, is freedom. We must guard the freedom to enhance public health from the regulatory creep of the narrowly–focused bureaucrat and the centralized planner for the average health of populations. That freedom was best expressed by Albert Camus: "Freedom is the right to take risks and excel. The status quo and good enough enslave."

50-235 97
97-139 97
A75998 169
A76154 174
A86719.1 105
ABT 418 46
AC-137 165
acardi 307
Accutane™ 120
acecol 311
acetylcholine receptor 41
acitretin 119
acne 119,120,124
actarit 296
acute promyelocytic leukemia 121,122
N-acyl-homoserine lactones 117
adapalene (CD 271) 124
adenosine agonists 16
adhesion blockers 154
a-2 adrenoceptor agonists 14
ω-Aga-IVA 52,53,54,55
ω-Aga-IVB (ω-aga-TK) 54,55
ω-Aga-TK (ω-Aga-IVB) 54,55
ω-Agatoxin-IA 54
ω-Agatoxin-IIA 54
ω-Agatoxin-IIIA 54
ω-agatoxins 54
AGN190,168 (tazarotene) 124
κ agonists 12
δ agonists 12
μ agonists 11
AIDS dementia 37
AL0670 84
AL0671 86
ALEC 308
alesion 299
aliphatic alcohols 57
Alzheimer's disease 36,42
AM 580 123
AM 80 123
AM-1155 104
aminoethoxy fluorochromans 6
aminoglycosides 55
amitryptyline 56
amoxicillin 152
ampicillin 152
ampiroxicam 296
amylin 165
anabaseine 42,46
anatoxin-a 44
angiopeptin 66
angiotensin II 268,296
angiotensin-converting enzyme 66
aniracetam 57
antarelix 173
antebate 297
antevate 297
17E8 antibody 258,259
1F7 antibody 260
29G11 antibody 259
43C9 antibody 257,258
antide 169,176
antiglaucoma 312
antiinflammatory agents 66
antiplatelet drugs 66
antisense oligonucleotides 67
AP-1 121,123
apacillin 103
apoptosis 121,125,126
aprikalim 81,82,84
argatroban 72
aryloxypropanolamines 6
arylpiperazines 7
L-asparaginase 306
astat 302
atherectomy 63
atherosclerosis 61
atrial natriuretic peptide 266
autophosphatase 116
autophosphorylation 116
azalines A and B 172
azidothymidine (AZT) 320
azithromycin 106
AZT (azidothymidine) 320
bafilomycin 156
Bak 243
balhimycin 107
balloon angioplasty 62
Basen 313
Bay K 8644 56
Bay y 3118 105
Bcl-2 243
BCR/ABL 250
bis-benzylisoquinoline alkaloids 55
betamethasone butyrate propionate 297
BG-8967 73
biodegradable polymers 67
BM-180680 103
BMS-180448 86
BMS-181139 103
BMS-182874 96
BMY 44621 75
BO-2727 102
BP0244 84
BQ-123 91,93,94,97,98
BQ-3020 91
BQ-4269 95
bradykinin 16,269
bradykinin antagonists 16
brimonidine (UK 14,304) 14
BRL 26,830 189
BRL 28,410 189
BRL 35,135 189
BRL 37,344 189
BRL 49653 162
BRL 55834 86
buspirone 1
BW373U86 12
calcicludine 55
calcipeptin 52
calcium channels 66
Campto 301
cancer chemotherapy 119,121,126
cannabinoids 14
carbapenem 303
carbapenems 102,103
Carbenin 306
carcinogenesis 121,122
cardiotonic vasodilator 307
L-carnosine 308
catheter-delivered genes 221
CD 1530 124
CD 271 (Adapalene) 124
CD 437 124
CD 495 123
CD 564 123
CD 666 124
cefditoren pivoxil 297
Cerezyme 301
cervical cancer 122

cetrorelix 169,176
CFTR gene 220
CGP 12177 190
CGRP(8-37) 165
ω-CgTx-GVIA 52,53,54
chlorpromazine 56
cholecystokinin antagonists 16
cholinergic agents 15
chorismic acid 260
ciglitazone 161
ciprofloxacin 104
CL 316,243 194
CL-186195 103
CL-191121 103
clarithromycin 106,152
clinafloxacin 104
clomipramine 56
ω-CmTx-MVIIA (SNX-111) 52,53,54
ω-CmTx-MVIIC 52,53,54
ω-CmTx-MVIID 53
CNJ206 antibody 259
CNS 1237 57
colchicine 66
compactin 214
complement cascade 35
ω-conotoxins 53,54
corticotropin-releasing factor (CRF) 21
cotransfection assays 120,123
Cozaar 302
CP-116517 104
CP-99219 104
CRF receptor antagonists 27
CRF binding protein 24
cromakalim 81,82
CS-045 161
CTLA4Ig 205
CVS-1123 75
CVS-995 76
cyclic hexapeptides 265
cyclooxygenase inhibitors 179
cyclosporin-A 265
cyclothialidine (Ro 09-1437) 107
cystic fibrosis 220,298
cytisine 42
cytokine induction 133,135,136
cytokines 31,32,33,34
daurisoline 55
DCCT (Diabetes Control and ComplicationsTrial) 159
Delivert 297
desipramine 56
deslorelin 171
detirelix 169,176
dexamethasone 214
dexibuprofen 298
Diabetes Control and Complications Trial(DCCT) 159
diazoxide 81,82,84
1,25-dihydroxy vitamin D3 214
diltiazem 56
dipalmitoylphosphatidylcholine 308
dirithromycin 106
Disopain 304
DMG-DMDOT 106
DMG-Mino 106
DNA gyrase inhibitors 104,105,107
docarpamine 298
domase alfa 298
drug delivery catheters 65,67
DU-6859a 104
DuP 714 75
DX-9065a 78
E1077 101
E1100 101
E1101 101
efegatran 74
Effexor 312
efonidipine 299
eliprodil 57
emopamil 56
ENA-78 209,210
endarterectomy 64
englitazone 161,161
epibatidine 15,43
epidermal growth factor 280
epidermal growth factor receptor 247
epinastine 299
eremomycin 107
Erk 249
erthromycin 113
Esmeron 309
ETH615 215
etretinate 119,120,122
FADD 242
famciclovir 300
Famvir 300
fantofarone (SR 33557) 56
FAP-1 242
fenretinide (4-HRP) 119,122
fish oils 66
FK037 101
flesinoxan 1,2,3
Flucam 296
flunarizine 56
fluoxetine 3
fluphenazine 56
fluspirilene 56
fluticasone 214
fluvastatin 300
FR139317 91,97
FTX 55
s-FTX 3.3 52
galanin 15
galanin ligands 15
ganiciclovir 221
ganirelix 169,176
gastric cancer 151
Gaucher's disease 301
gene therapy 68,219
GHRP-6 270
GLP-1 (glucagon-like peptide-1) 160
glucagon-like peptide-1 (GLP-1) 160
glucan clinical data 134,135,136
glucan receptors 130,131
glucan structure 129,130,135
glycopeptides 107
glycoprotein IIb/IIIa receptor antibody 66
glycylcyclines 105,106
GM-CSF 280
gold sodium thiomalate 214
grafts 64
ω-Grammatoxin SIA 55
Grb2 247
GRO 209,210
growth hormone (hGH) 278
growth hormone insensitivity 310
gusperimus 300
GUSTO IIa 73
GV-118819 103
GYKI-14766 74

haloperidol 56
hammerhead ribozyme 288
HDV ribozyme 289
hemodialysis access grafts 64
heparin 66
hepatitis C virus protease 144
herpesvirus protease 145
hirudin 66,72
hirulog 66,73
HIT-III 73
HIV protease 139
HIV reverse transcriptase 146
HMG-CoA reductase inhibitor 300
5-HT1A agonists 1,2,3,4,5
5-HT1A antagonists 2,3,5,6,7
5-HT1A autoreceptors 3
5-HT1A receptors 1,2
5-HT_2 receptor antagonists 305
5-HT_3 receptor antagonists 305
human cancer 121
hyoscyamine 15
hyperprolactinemia 309
ICE 243
ICI 118,551 191
ICI 198157 194
ICI 201651 194
ICI D7114 194
idiopathic hypersomnia 303
ifenprodil 57
Igef 310
IL-8 209,210,211,213
imiglucerase 301
imipramine 56
immune response 114,115
immunomodulators 145
immunomodulatory 115
immunostimulatory 115
immunosuppressive 115
inogatran 76
insulin 280
interferon 32,33
interleukin-1β-converting enzyme 34
interleukin-2 (IL-2) 280
interleukin-5 (IL-5) 279
interleukin-6 (IL-6) 278
interleukin-8 (IL-8) 278
interleukins 32,33
intracellular receptors 120
intracellular signaling 116
invertebrate toxins 53
IPI-950 97
IRAP gene 222
irinotecan 301
irreversible proton pump inhibitor 306
izenamicin 107
kallikrein 16
KB-2796 56
KC-399 86
ketolides 106
ketoralac 11
Ki1769 84
Ki3315 84
KRM-1648 107
KRM-1657 107
KRN2391 84
L-695256 102
L-745,337 186
L-746,072 96
L-747,844 96
L-749,329 96
L-754,142 96
β-lactamase inhibitors 103
β-lactams 101,102,103
Landel 299
lanoconazole 302
Laron-type dwarfism 310
lentinan 129,130,134,136
Lescol 300
leucovorin 312
leukopenia 304
leuprolide 171
leveromakalim 82,87
levofloxacin 104
LGD1069 124,125,126
lipid-lowering drug 66
LK-10517 101
lobeline 42
loperamide 57
losartan 302
lovastatin 214
low dose radiation 67
lung surfactant 308
LY 104119 192
LY 79771 192
LY 287045 74
LY 293284 5
LY 294468 74
macrolides 106,107
bis(maltolato)oxovanadium(IV) 164
MAO inhibitors 3
MAPK 249
MCP-1 211,212,213
MCP-2 211,212,213
MCP-3 211,212,213
MD-805 72
MDL73756 75
medicinal chemists 329
Meiact 297
meptazinol 12
meropenem 303
Merrem 303
methotrexate 214
metocurine 43
metronidazole 152
3-MeTTNPB 124
midaglizole 159
mifepristone (RU486) 214
minoxidil 81,82,83
MIP-1α 211,212,213
MIP-1ß 211,212,213
mirtazapine 303
mixed opioid ligands 13
MK-329 16
moclobemide 3
modafinil 303
Modiodal 303
mofezolac 304
Mover 296
MT 151 204
MTP-1403 160
multiple sclerosis 37,38
Nacyl 296
nafarelin 171
naglivan 164
naltrindole 13
NAN-190 3
narcolepsy 303
nartograstim 304
nazasetron 305
NC-1100 56

nefazodone 305
neomycin 55
Neu-Up 304
neuroleptics 56
neuromuscular blocker 309
NeuTrexin 312
NGF 249
nicorandil 81,82,84,85
nicotine 42,45,47
nicotinic acetylcholine receptor 41
NIDDM (non-insulin-dependent diabetes) 159
nitric oxide synthetase 66
NM394 104
NMDA antagonists 57
NNC 09-0026 56
non-antibiotic 114
non-insulin-dependent diabetes (NIDDM) 159
non-opioid analgesics 14
Norprolac 309
NPA-2 209,210
NPC-567 16
NS004 85
NS1619 85
Nutropin 310
ofloxacin 104
8-OH-DPAT 1,4
OKT 3 201
OKT 8 204
oleandomycin 106
omeprazole 152
Oncaspar 306
OPC-17116 104
opioid analgesics 11
oral activity of glucans 133
oral leukoplakia 121,122
Orcl 296
ornithine decarboxylase (ODC) 121,123
P13K 229
panipenem/betamipron 305
pantoprazole 306
Parkinson's disease 42,222
paroxetine 3
pathogen 112,115
PD 131682 104
PD 138312 104
PD 140248 104
PD 142893 93,94
PD 145065 93
PD 155080 95
PD 156707 95
pegaspargase 306
Perazolin 310
percutaneous transluminal coronaryangioplasty (PTCA) 62
PGG-glucan (Betafectin) 134,135
pharmacology of glucans 132,133,134
phosphotransferase 116
photodynamic therapy 67
PhRMA 339
Pimenol 307
pimobendan 307
pimozide 56
pinacidil 81,82,83
pindobind 4
pindolol 3,4
pioglitazone 161
pirmenol 307
platelet-derived growth factor 66
Pneumocystis carinii pneumonia 312
polaprezinc 307
PPACK 74
prephenic acid 260
progesterone 214
Promac 307
propagermanium 308
(-)-propranolol 3
prostacyclin 66
proteoglycans 215
psoriasis 119,120,124
Pulmozyme 298
pumactant 308
bis(pyrrolidine-N-carbodithioato)oxo-vanadium(IV) 164
Q-35 104
quinagolide 309
quinolones 104,105
R 56865 57
Raf 249
ranitidine 152
RANTES 211,212,213
Ras 248
Remeron 303
remifentanil 11
RES-701-1 94
Rescula 312
resistance 111
restenosis 61,221
Retin A™ 120
retinoic acid, all-*trans* (ATRA) 119,120,121,122,123,124,125
retinoic acid, 13-*cis* (13-*cis* RA) 119,120,121,122
retinoic acid, 9-*cis* (9-*cis*-RA) 119,120,121,122,124,125,126
retinoic acid receptors (RARs) 120,121,124,126
retinoid 119,120,121,122,123,124,125,126
retinoid receptors 119,120
retinoid X receptors (RXRs) 120,124,126
RH-5529 57
8RN-DAGal 55
Ro 09-1437 (cyclothialidine) 107
Ro 16-8714 192
Ro 40-2148 192
Ro 41-5253 125
Ro 46-2005 93
Ro 46-2548 76
Ro 46-5471 125
Ro 46-6240 76
Ro 46-8515 125
Ro 47-0203, bosentan, Laroteline® 91,93,94
Ro-48-1220 103
rocuronium bromide 309
rolipram 34
roxithromycin 106
RP49356 82
RP66266 84
RP66784 84
RU-59863 101
RU486 214,223
rufloxacin 104
S 14489 7
S 15535 7
S 15931 7
S-4661 103
S15261 163
salmon calcitonin 165
sartide 172
SB 201823-A 57
SB209670 94,95
SB209834 95

SB217242 95
SC-57666 183
SC-58125 183
SC-58231 184
SC-58451 185
SCA40 85
schizophyllan (Sizofiran) 129,136
scleroglucan 129,132,133,134
SDZ-PCO-400 86
SDZ217766 76
SDZ219349 76
selective serotonin reuptake inhibitors 3
Seractil 298
Serosion 308
Serotone 305
serotonergic ligands 16
serotonin (5-HT) reuptake inhibitor 305
serotonin receptor 66
serotonin receptors 1
sertraline 3
Serzone 305
SH-PTP2 229
SK&F 96365 57
SL 84.0418 160
SMANCS 313
smooth muscle cells 61
SNC 80 12
SNX-111 (ω-CmTx-MVIIA) 54
sobuzoxane 310
somatomedin-1 310
somatotropin 310
Sos 248
Spanidin 301
sparfloxacin 104
spider venoms 55
spiperone 56
SR 33557 (fantofarone) 56
SR 58894A 190
SR 59203A 190
SR-59062 191
SR11217 124,126
SR11237 124
SR47063 83
stavudine 311
stents 64
sub-MICs 113
Substance P 16
subtractive hybridization 114
sumatriptan 16
suramin 35
T-159 173
T-3761 104
tachykinin antagonists 16
TAK-044 97,98
Tanadopa 298
tazarotene (AGN190,168) 124
TBG-Mino 106
TCV-295 85
TD550 125
TD650 125
Tegison™ 120
temocapril 311
(-)-tertatolol 6
tetracycline resistance 105
tetracyclines 105,106
tetrandrine 55
thalidomide 319
theophylline 281
thiondazine 56
thrombolytics 66
thromboxane A2 synthetase 66
thromboxane receptors 66
TIMI 9A 73
tissue transglutaminase (Tgase) 121,125
TNF 33,34
TNFα (tumor necrosis factor α) 320
Topotecin 301
toxoplasmosis 312
tramadol 11,13
transcription factors 120
transplant atherosclerosis 64
tricyclic antidepressants 56
trimetrexate 312
triple therapy 154
troglitazone 161
TTAB 123
TTNN 123
TTNPB 123,124,125
tumor necrosis factor α (TNFα) 320
two-component switch 116
tylosin 107
tyrosine kinase 247
U-89232 86
(S)-UH-301 2
UK 14,304 (brominodine) 14
UR8225 83
urease inhibitors 155
vaccines 113
vanadate 163
vancomycin 112
venlafaxine 312
verapamil 56
viral protease 139
virulence 111
vitamin A 119,120,121
voglibose 313
(S)-WAY-100135 2,7
WAY-100635 7
wound healing 62
YM934 86
ZAP-70 229
ZD2079 193
ZD6169 87
ZD7114 194
Zemuron 309
Zerit 311
zinostatin stimalamer 313

acetylcholine receptors, 30, 41
acetylcholine transporter, 28, 247
adenylate cyclase, 6, 227, 233; 12, 172; 19, 293; 29, 287
adenosine, neuromodulator, 18, 1; 23, 39
adjuvants, 9, 244
adrenal steroidogenesis, 2, 263
ß-adrenergic blockers, 10, 51; 14, 81
affinity labeling, 9, 222
$ß_3$-agonists, 30, 189
AIDS, 23, 161, 253; 25, 149
alcohol consumption, drugs and deterrence, 4, 246
aldose reductase, 19, 169
alkaloids, 1, 311; 3, 358; 4, 322; 5, 323; 6, 274
allergy, 29, 73
alopecia, 24, 187
Alzheimer's Disease, 26, 229; 28, 49, 197, 247
aminocyclitol antibiotics, 12, 110
amyloid, 28, 49
amyloidogenesis, 26, 229
analgesics (analgetic), 1, 40; 2, 33; 3, 36; 4, 37; 5, 31; 6, 34; 7, 31; 8, 20; 9, 11; 10, 12; 11, 23; 12, 20; 13, 41; 14, 31; 15, 32; 16, 41; 17, 21; 18, 51; 19, 1; 20, 21; 21, 21; 23, 11; 25, 11; 30, 11
androgen action, 21, 179; 29, 225
anesthetics, 1, 30; 2, 24; 3, 28; 4, 28; 7, 39; 8, 29; 10, 30
angiogenesis, 27, 139
angiotensin/renin modulators, 26, 63; 27, 59
animal engineering, 29, 33
animal models, anxiety, 15, 51
animal models, memory and learning, 12, 30
Annual Reports in Medicinal Chemistry, 25, 333
anorexigenic agents, 1, 51; 2, 44; 3, 47; 5, 40; 8, 42; 11, 200; 15, 172
antagonists, calcium, 16, 257; 17, 71; 18, 79
antagonists, GABA, 13, 31; 15, 41
antagonists, narcotic, 7, 31; 8, 20; 9, 11; 10, 12; 11, 23
antagonists, non-steroidal, 1, 213; 2, 208; 3, 207; 4, 199
antagonists, steroidal, 1, 213; 2, 208; 3, 207; 4, 199
anthracycline antibiotics, 14, 288
antiaging drugs, 9, 214
antiallergy agents, 1, 92; 2, 83; 3, 84; 7, 89; 9, 85; 10, 80; 11, 51; 12, 70; 13, 51; 14, 51; 15, 59; 17, 51; 18, 61; 19, 93; 20, 71; 21, 73; 22, 73; 23, 69; 24, 61; 25, 61; 26, 113; 27, 109
antianginals, 1, 78; 2, 69; 3, 71; 5, 63; 7, 69; 8, 63; 9, 67; 12, 39; 17, 71
antianxiety agents, 1, 1; 2, 1; 3, 1; 4, 1; 5, 1; 6, 1; 7, 6; 8, 1; 9, 1; 10, 2; 11, 13; 12, 10; 13, 21; 14, 22; 15, 22; 16, 31; 17, 11; 18, 11; 19, 11; 20, 1; 21, 11; 22, 11; 23, 19; 24, 11
antiarrhythmics, 1, 85; 6, 80; 8, 63; 9, 67; 12, 39; 18, 99, 21, 95; 25, 79; 27, 89
antibacterial agents, 1, 118; 2, 112; 3, 105; 4, 108; 5, 87; 6, 108; 17, 107; 18, 109; 19, 107; 20, 145, 155; 21, 139; 23, 133; 24, 101; 25, 109; 26, 123; 27, 119; 29, 113; 30, 101
antibacterial agents, novel approaches, 23, 141
antibacterial resistance mechanisms, 28, 141
antibiotic transport, 24, 139
antibiotics, 1, 109; 2, 102; 3, 93; 4, 88; 5, 75, 156; 6, 99; 7, 99, 217; 8, 104; 9, 95; 10, 109, 246; 11, 89; 11, 271; 12, 101, 110; 13, 103, 149; 14, 103; 15, 106; 17, 107; 18, 109; 21, 131; 23, 121; 24, 101; 25, 119
antibiotic producing organisms, 27, 129
antibodies, cancer therapy, 23, 151

antibodies, drug carriers and toxicity reversal, 15, 233
antibodies, monoclonal, 16, 243
anticancer agents, mechanical-based, 25, 129
anticancer drug resistance, 23, 265
anticonvulsants, 1, 30; 2, 24; 3, 28; 4, 28; 7, 39, 8, 29; 10, 30; 11, 13; 12, 10; 13, 21; 14, 22; 15, 22; 16, 31; 17, 11; 18, 11; 19, 11; 20, 11; 21, 11; 23, 19; 24, 11
antidepressants, 1, 12; 2, 11; 3, 14; 4, 13; 5, 13; 6, 15; 7, 18; 8, 11; 11, 3; 12, 1; 13, 1; 14, 1; 15, 1; 16, 1; 17, 41; 18, 41; 20, 31; 22, 21; 24, 21; 26, 23; 29, 1
antidiabetics, 1, 164; 2, 176; 3, 156; 4, 164; 6, 192; 27, 219
antifungals, 2, 157; 3, 145; 4, 138; 5, 129; 6, 129; 7, 109; 8, 116; 9, 107; 10, 120; 11, 101; 13, 113; 15, 139; 17, 139; 19, 127; 22, 159; 24, 111; 25, 141; 27, 149
antiglaucoma agents, 20, 83
antihyperlipidemics, 15, 162; 18, 161; 24, 147
antihypertensives, 1, 59; 2, 48; 3, 53; 4, 47; 5, 49; 6, 52; 7, 59; 8, 52; 9, 57; 11, 61; 12, 60; 13, 71; 14, 61; 15, 79; 16, 73; 17, 61; 18, 69; 19, 61; 21, 63; 22, 63; 23, 59; 24, 51; 25, 51
antiinfective agents, 28, 119
antiinflammatory agents, 28, 109; 29, 103
antiinflammatories, non-steroidal, 1, 224; 2, 217; 3, 215; 4, 207; 5, 225; 6, 182; 7, 208; 8, 214; 9, 193; 10, 172; 13, 167; 16, 189; 23, 181
anti-ischemic agents, 17, 71
antimetabolite concept, drug design, 11, 223
antimicrobial drugs - clinical problems and opportunities, 21, 119
antimicrobial peptides, 27, 159
antineoplastics, 2, 166; 3, 150; 4, 154; 5, 144; 7, 129; 8, 128; 9, 139; 10, 131; 11, 110; 12, 120; 13, 120; 14, 132; 15, 130; 16, 137; 17, 163; 18, 129; 19, 137; 20, 163; 22, 137; 24, 121; 28, 167
antiparasitics, 1, 136, 150; 2, 131, 147; 3, 126, 140; 4, 126; 5, 116; 7, 145; 8, 141; 9, 115; 10, 154; 11, 121; 12, 140; 13, 130; 14, 122; 15, 120; 16, 125; 17, 129; 19, 147; 26, 161
antiparkinsonism drugs, 6, 42; 9, 19
antipsychotics, 1, 1; 2, 1; 3, 1; 4, 1; 5, 1; 6, 1; 7, 6; 8, 1; 9, 1; 10, 2; 11, 3; 12, 1; 13, 11; 14, 12; 15, 12; 16, 11; 18, 21; 19, 21; 21, 1; 22, 1; 23, 1; 24, 1; 25, 1; 26, 53; 27, 49; 28, 39
antiradiation agents, 1, 324; 2, 330; 3, 327; 5, 346
anti-retroviral chemotherapy, 25, 149
antirheumatic drugs, 18, 171
antisense ologonucleotides, 23, 295
antisense technology, 29, 297
antithrombotics, 7, 78; 8, 73; 9, 75; 10, 99; 12, 80; 14, 71; 17, 79; 27, 99
antithrombotic agents, 29, 103
antitumor agents, 24, 121
antiviral agents, 1, 129; 2, 122; 3, 116; 4, 117; 5, 101; 6, 118; 7, 119; 8, 150; 9, 128; 10, 161; 11, 128; 13, 139; 15, 149; 16, 149; 18, 139; 19, 117; 22, 147; 23, 161; 24, 129; 26, 133; 28, 131; 29, 145; 30, 139
anxiolytics, 26, 1
aporphine chemistry, 4, 331
arachidonate lipoxygenase, 16, 213
arachidonic acid cascade, 12, 182; 14, 178
arachidonic acid metabolites, 17, 203; 23, 181; 24, 71
arthritis, 13, 167; 16, 189; 17, 175; 18, 171; 21, 201; 23, 171, 181
arthritis, immunotherapy, 23, 171
asthma, 29, 73
asymmetric synthesis, 13, 282
atherosclerosis, 1, 178; 2, 187; 3, 172; 4, 178; 5, 180; 6, 150; 7, 169; 8, 183; 15, 162; 18, 161; 21, 189; 24, 147; 25, 169; 28, 217
atrial natriuretic factor, 21, 273; 23, 101

autoreceptors, 19, 51
ß-lactam antibiotics, 23, 121; 24, 101
bacterial adhesins, 26, 239
bacterial resistance, 13, 239; 17, 119
bacterial toxins, 12, 211
bacterial virulence, 30, 111
basophil degranulation, biochemistry, 18, 247
behavior, serotonin, 7, 47
benzodiazepine receptors, 16, 21
bioisosterism, 21, 283
biological factors, 10, 39; 11, 42
biological membranes, 11, 222
biopharmaceutics, 1, 331; 2, 340; 3, 337; 4, 302; 5, 313; 6, 264; 7, 259; 8, 332
biosensor, 30, 275
biosynthesis, antibotics, 12, 130
biotechnology, drug discovery, 25, 289
blood-brain barrier, 20, 305
blood enzymes, 1, 233
bone, metabolic disease, 12, 223; 15, 228; 17, 261; 22, 169
bone metabolism, 26, 201
brain, decade of, 27, 1
calcium antagonists/modulators, 16, 257; 17, 71; 18, 79; 21, 85
calcium channels, 30, 51
calmodulin antagonists, SAR, 18, 203
cancer, 27, 169
cancer chemotherapy, 29, 165
cancer, drug resistance, 23, 265
cancer therapy, 2, 166; 3, 150; 4, 154; 5, 144; 7, 129; 8, 128; 9, 139, 151; 10, 131; 11, 110; 12, 120; 13, 120; 14, 132; 15, 130; 16, 137; 17, 163; 18, 129; 21, 257; 23, 151
cannabinoids, 9, 253
carbohydrates, 27, 301
carboxylic acid, metalated, 12, 278
carcinogenicity, chemicals, 12, 234
cardiotonic agents, 13, 92; 16, 93; 19, 71
cardiovascular agents, 10, 61
catalysis, intramolecular, 7, 279
catalytic antibodies, 25, 299; 30, 255
cell adhesion, 29, 215
cell adhesion molecules, 25, 235
cell based mechanism screens, 28, 161
cell invasion, 14, 229
cell metabolism, 1, 267
cell metabolism, cyclic AMP, 2, 286
cellular responses, inflammatory, 12, 152
chemokines, 30, 209
chemotaxis, 15, 224; 17, 139, 253; 24, 233
cholecystokinin, 18, 31
cholecystokinin agonists, 26, 191
cholecystokinin antagonists, 26, 191
chronopharmacology, 11, 251
circadian processes, 27, 11
coagulation, 26, 93
cognition enhancers, 25, 21
cognitive disorders, 19, 31; 21, 31; 23, 29
collagenase, biochemistry, 25, 177
collagenases, 19, 231

colony stimulating factor, 21, 263
complement cascade, 27, 199
complement inhibitors, 15, 193
complement system, 7, 228
conformation, nucleoside, biological activity, 5, 272
conformation, peptide, biological activity, 13, 227
conformational analysis, peptides, 23, 285
congestive heart failure 22, 85
contrast media, NMR imaging, 24, 265
corticotropin-releasing factor, 25, 217; 30, 21
cotransmitters, 20, 51
cyclic AMP, 2, 286; 6, 215; 8, 224; 11, 291
cyclic GMP, 11, 291
cyclic nucleotides, 9, 203; 10, 192; 15, 182
cyclooxygenase, 30, 179
cystic fibrosis, 27, 235
cytochrome P-450, 9, 290; 19, 201
cytokine, 27, 209
cytokine receptors, 26, 221
database searching, 3D, 28, 275
DDT-type insecticides, 9, 300
dermal wound healing, 24, 223
dermatology and dermatological agents, 12, 162; 18, 181; 22, 201; 24, 177
designer enzymes, 25, 299
diabetes, 9, 182; 11, 170; 13, 159; 19, 169; 22, 213; 25, 205; 30, 159
Diels-Alder reaction, intramolecular, 9, 270
distance geometry, 26, 281
diuretic, 1, 67; 2, 59; 3, 62; 6, 88; 8, 83; 10, 71; 11, 71; 13, 61; 15, 100
DNA binding, sequence-specific, 27, 311; 22, 259
docking strategies, 28, 275
dopamine, 13, 11; 14, 12; 15, 12; 16, 11, 103; 18, 21; 20, 41; 22, 107
dopamine D_3, 29, 43
dopamine D_4, 29, 43
drug abuse, CNS agents, 9, 38
drug allergy, 3, 240
drug carriers, antibodies, 15, 233
drug carriers, liposomes, 14, 250
drug delivery systems, 15, 302; 18, 275; 20, 305
drug design, metabolic aspects, 23, 315
drug discovery, natural sources, 17, 301
drug disposition, 15, 277
drug metabolism, 3, 227; 4, 259; 5, 246; 6, 205; 8, 234; 9, 290; 11, 190; 12, 201; 13, 196, 304; 14, 188; 16, 319; 17, 333; 23, 265, 315; 29, 307
drug receptors, 25, 281
drug resistance, 23, 265
EDRF, 27, 69
elderly, drug action, 20, 295
electrosynthesis, 12, 309
enantioselectivity, drug metabolism, 13, 304
endorphins, 13, 41; 14, 31; 15, 32; 16, 41; 17, 21; 18, 51
endothelin antagonists, 29, 65, 30, 91
enzymatic monooxygenation reactions, 15, 207
enzyme inhibitors, 7, 249; 9, 234; 13, 249
enzyme immunoassay, 18, 285
enzymes, anticancer drug resistance, 23, 265
enzymes, blood, 1, 233
enzymes, proteolytic inhibition, 13, 261

enzyme structure-function, 22, 293
enzymic synthesis, 19, 263; 23, 305
epitopes for antibodies, 27, 189
ethnobotany, 29, 325
excitatory amino acids, 22, 31; 24, 41; 26, 11; 29, 53
fertility control, 10, 240; 14, 168; 21, 169
forskolin, 19, 293
free radical pathology, 10, 257; 22, 253
G-proteins, 23, 235
GABA, antagonists, 13, 31; 15, 41
gamete biology, fertility control, 10, 240
gastrointestinal agents, 1, 99; 2, 91; 4, 56; 6, 68; 8, 93; 10, 90; 12, 91; 16, 83; 17, 89; 18, 89; 20,117; 23, 201
gene expression, inhibitors, 23, 295
gene targeting technology, 29, 265
gene therapy, 8, 245; 30, 219
gene transcription, regulation of, 27, 311
glucagon, mechanism, 18, 193
ß-D-glucans, 30, 129
glucocorticosteroids, 13, 179
glycoconjugate vaccines, 28, 257
glycoprotein IIbIIIa antagonists, 28, 79
glycosylation, non-enzymatic, 14, 261
gonadotropin releasing hormone, 30, 169
growth factors, 21, 159; 24, 223; 28, 89
growth hormone, 20, 185
growth hormone secretagogues, 28, 177
guanylyl cyclase, 27, 245
hallucinogens, 1, 12; 2, 11; 3, 14; 4, 13; 5, 23; 6, 24
heart disease, ischemic, 15, 89; 17, 71
heart failure, 13, 92; 16, 93; 22, 85
helicobacter pylori, 30, 151
hemorheologic agents, 17, 99
herbicides, 17, 311
heterocyclic chemistry, 14, 278
HIV protease inhibitors, 26, 141; 29, 123
HIV reverse transcriptase inhibitors, 29, 123
HIV vaccine, 27, 255
homeobox genes, 27, 227
hormones, glycoprotein, 12, 211
hormones, non-steroidal, 1, 191; 3, 184
hormones, peptide, 5, 210; 7, 194; 8, 204; 10, 202; 11, 158; 16, 199
hormones, steroid, 1, 213; 2, 208; 3, 207; 4, 199
host modulation, infection, 8, 160; 14, 146; 18, 149
human gene therapy, 26, 315; 28, 267
human retrovirus regulatory proteins, 26, 171
5-hydroxytryptamine, 2, 273; 7, 47; 21, 41
hypercholesterolemia, 24, 147
hypersensitivity, delayed, 8, 284
hypersensitivity, immediate, 7, 238; 8, 273
hypertension, 28, 69
hypertension, etiology, 9, 50
hypnotics, 1, 30; 2, 24; 3, 28; 4, 28; 7, 39; 8, 29; 10, 30; 11, 13; 12, 10; 13, 21; 14, 22; 15, 22, 16; 31; 17, 11; 18, 11; 19, 11; 22, 11
IgE, 18, 247
immune mediated idiosyncratic drug hypersensitivity, 26, 181
immunity, cellular mediated, 17, 191; 18, 265

immunoassay, enzyme, 18, 285
immunophilins, 28, 207
immunostimulants, arthritis, 11, 138; 14, 146
immunosuppressants, 26, 211; 29, 175
immunosuppressive drug action, 28, 207
immunosuppressives, arthritis, 11, 138
immunotherapy, cancer, 9, 151; 23, 151
immunotherapy, infectious diseases, 18, 149; 22, 127
immunotherapy, inflammation, 23, 171
infections, sexually transmitted, 14, 114
inflammation, 22, 245
inflammation, immunomodulatory approaches, 23, 171
inflammation, proteinases in, 28, 187
inflammatory bowel disease, 24, 167
inhibitors, complement, 15, 193
inhibitors, connective tissue, 17, 175
inhibitors, enzyme, 13, 249
inhibitors, irreversible, 9, 234; 16, 289
inhibitors, platelet aggregation, 6, 60
inhibitors, proteolytic enzyme, 13, 261
inhibitors, renin-angiotensin, 13, 82
inhibitors, reverse transcription, 8, 251
inhibitors, transition state analogs, 7, 249
inorganic chemistry, medicinal, 8, 294
inositol triphosphate receptors, 27, 261
insecticides, 9, 300; 17, 311
insulin, mechanism, 18, 193
interferon, 8, 150; 12, 211; 16, 229; 17, 151
interleukin-1, 20, 172; 22, 235; 25, 185; 29, 205
interleukin-2, 19, 191
interoceptive discriminative stimuli, animal model of anxiety, 15, 51
intramolecular catalysis, 7, 279
ion channels, ligand gated, 25, 225
ion channels, voltage-gated, 25, 225
ionophores, monocarboxylic acid, 10, 246
iron chelation therapy, 13, 219
irreversible ligands, 25, 271
ischemia/reperfusion, CNS, 27, 31
ischemic injury, CNS, 25, 31
isotopes, stable, 12, 319; 19, 173
ß-lactam antibiotics, 11, 271; 12, 101; 13, 149; 20, 127, 137; 23, 121
ß-lactamases, 13, 239; 17, 119
learning, 3, 279; 16, 51
leukocyte elastase inhibitors, 29, 195
leukocyte motility, 17, 181
leukotrienes, 17, 291; 19, 241; 24, 71
LHRH, 20, 203; 23, 211
lipid metabolism, 9, 172; 10, 182; 11, 180; 12, 191; 13, 184; 14, 198; 15, 162
lipoproteins, 25, 169
liposomes, 14, 250
lipoxygenase, 16, 213; 17, 203
lymphocytes, delayed hypersensitivity, 8, 284
macrolide antibiotics, 25, 119
macrocyclic immunomodulators, 25, 195
magnetic resonance, drug binding, 11, 311
managed care, 30, 339

market introductions, 19, 313; 20, 315; 21, 323; 22, 315; 23, 325; 24, 295; 25, 309
mass spectrometry, of peptides, 24, 253
mass spectrometry, tandem, 21, 213; 21, 313
mast cell degranulation, biochemistry, 18, 247
mechanism based, anticancer agents, 25, 129
mechanism, drug allergy, 3, 240
mechanisms of antibiotic resistance, 7, 217; 13, 239; 17, 119
medicinal chemists, 30, 329
medicinal chemistry, 28, 343
membrane function, 10, 317
membrane regulators, 11, 210
membranes, active transport, 11, 222
memory, 3, 279; 12, 30; 16, 51
metabolism, cell, 1, 267; 2, 286
metabolism, drug, 3, 227; 4, 259; 5, 246; 6, 205; 8, 234; 9, 290; 11, 190; 12, 201; 13, 196, 304; 14, 188; 23, 265, 315
metabolism, lipid, 9, 172; 10, 182; 11, 180; 12, 191; 14, 198
metabolism, mineral, 12, 223
metal carbonyls, 8, 322
metals, disease, 14, 321
metastasis, 28, 151
microbial products screening, 21, 149
migraine, 22, 41
mitogenic factors, 21, 237
modified serum lipoproteins, 25, 169
molecular diversity, 26, 259, 271; 28, 315
molecular modeling, 22, 269; 23, 285
monoclonal antibodies, 16, 243; 27, 179; 29, 317
monoclonal antibody cancer therapies, 28, 237
monoxygenases, cytochrome P-450, 9, 290
muscarinic agonists/antagonists, 23, 81; 24, 31; 29, 23
muscle relaxants, 1, 30; 2, 24; 3, 28; 4, 28; 8, 37
muscular disorders, 12, 260
mutagenicity, mutagens, 12, 234
mutagenesis, SAR of proteins, 18, 237
myocardial ischemia, acute, 25, 71
narcotic antagonists, 7, 31; 8, 20; 9, 11; 10, 12; 11, 23; 13, 41
natriuretic agents, 19, 253
natural products, 6, 274; 15, 255; 17, 301; 26, 259
natural killer cells, 18, 265
neoplasia, 8, 160; 10, 142
neurodegeneration, 30, 31
neurodegenerative disease, 28, 11
neurokinin receptor antagonists, 26, 43
neuronal calcium channels, 26, 33
neuronal cell death, 29, 13
neuropeptides, 21, 51; 22, 51
neuroprotection, 29, 13
neurotensin, 17, 31
neurotransmitters, 3, 264; 4, 270; 12, 249; 14, 42; 19, 303
neutrophic factors, 25, 245; 28, 11
neutrophil chemotaxis, 24, 233
nicotinic acetylcholine receptor, 22, 281
nitric oxide synthase, 29, 83
NMR, 27, 271
NMR in biological systems, 20, 267

NMR imaging, 20, 277; 24, 265
NMR, protein structure determination, 23, 275
non-enzymatic glycosylation, 14, 261
non-nutritive, sweeteners, 17, 323
non-steroidal antiinflammatories, 1, 224; 2, 217; 3, 215; 4, 207; 5, 225; 6, 182; 7, 208; 8, 214; 9, 193; 10, 172; 13, 167; 16, 189
nucleic acid-drug interactions, 13, 316
nucleic acid, sequencing, 16, 299
nucleic acid, synthesis, 16, 299
nucleoside conformation, 5, 272
nucleosides, 1, 299; 2, 304; 3, 297; 5, 333
nucleotide metabolism, 21, 247
nucleotides, 1, 299; 2, 304; 3, 297; 5, 333
nucleotides, cyclic, 9, 203; 10, 192; 15, 182
obesity, 1, 51; 2, 44; 3, 47; 5, 40; 8, 42; 11, 200; 15, 172; 19, 157; 23, 191
oligonucleotides, inhibitors, 23, 295
oncogenes, 18, 225; 21, 159, 237
opioid receptor, 11, 33; 12, 20; 13, 41; 14, 31; 15, 32; 16, 41; 17, 21; 18, 51; 20, 21; 21, 21
opioids, 12, 20; 16, 41; 17, 21; 18, 51; 20, 21; 21, 21
opportunistic infections, 29, 155
organocopper reagents, 10, 327
osteoarthritis, 22, 179
osteoporosis, 22, 169; 26, 201; 29, 275
P-glycoprotein, multidrug transporter, 25, 253
parasite biochemistry, 16, 269
patents in medicinal chemistry, 22, 331
pathophysiology, plasma membrane, 10, 213
penicillin binding proteins, 18, 119
peptic ulcer, 1, 99; 2, 91; 4, 56; 6, 68; 8, 93; 10, 90; 12, 91; 16, 83; 17, 89; 18, 89; 19, 81; 20, 93; 22, 191; 25, 159
peptide conformation, 13, 227; 23, 285
peptide hormones, 5, 210; 7, 194; 8, 204; 10, 202; 11, 158, 19, 303
peptide hypothalamus, 7, 194; 8, 204; 10, 202; 16, 199
peptide libraries, 26, 271
peptide receptors, 25, 281
peptide, SAR, 5, 266
peptide stability, 28, 285
peptide synthesis, 5, 307; 7, 289; 16, 309
peptide synthetic, 1, 289; 2, 296
peptide thyrotropin, 17, 31
peptidomimetics, 24, 243
periodontal disease, 10, 228
PET, 24, 277
pharmaceutics, 1, 331; 2, 340; 3, 337; 4, 302; 5, 313; 6, 254, 264; 7, 259; 8, 332
pharmacokinetics, 3, 227, 337; 4, 259, 302; 5, 246, 313; 6, 205; 8, 234; 9, 290; 11, 190; 12, 201; 13, 196, 304; 14, 188, 309; 16, 319; 17, 333
pharmacophore identification, 15, 267
pharmacophoric pattern searching, 14, 299
phosphodiesterase inhibitors, 29, 185
phospholipases, 19, 213; 22, 223; 24, 157
physicochemical parameters, drug design, 3, 348; 4, 314; 5, 285
pituitary hormones, 7, 194; 8, 204; 10, 202
plasma membrane pathophysiology, 10, 213
plasminogen activator, 18, 257; 20, 107; 23, 111
platelet activating factor (PAF), 17, 243; 20, 193; 24, 81
platelet aggregation, 6, 60

polyether antibiotics, 10, 246
polyamine metabolism, 17, 253
polyamine spider toxins, 24, 287
polymeric reagents, 11, 281
positron emission tomography, 24, 277, 25, 261
potassium channel activators, 26, 73
potassium channel antagonists, 27, 89
potassium channel openers, 24, 91, 30, 81
prodrugs, 10, 306; 22, 303
programmed cell death, 30, 239
prolactin secretion, 15, 202
prostacyclin, 14, 178
prostaglandins, 3, 290; 5, 170; 6, 137; 7, 157; 8, 172; 9, 162; 11, 80
prostatic disease, 24, 197
proteases, 28, 151
protein C, 29, 103
protein growth factors, 17, 219
proteinases, arthritis, 14, 219
protein kinases, 18, 213; 29, 255
protein kinase C, 20, 227; 23, 243
protein phosphatases, 29, 255
protein structure determination, NMR, 23, 275
protein tyrosine kinases, 27, 169
psoriasis, 12, 162
psychiatric disorders, 11, 42
psychoses, biological factors, 10, 39
psychotomimetic agents, 9, 27
pulmonary agents, 1, 92; 2, 83; 3, 84; 4, 67; 5, 55; 7, 89; 9, 85; 10, 80; 11, 51; 12, 70; 13, 51; 14, 51; 15, 59; 17, 51; 18, 61; 20, 71; 21, 73; 22, 73; 23, 69; 24, 61; 25, 61; 26, 113; 27, 109
quantitative SAR, 6, 245; 8, 313; 11, 301; 13, 292; 17, 281
quinolone antibacterials, 21, 139; 22, 117; 23, 133
radioimmunoassays, 10, 284
radioisotope labeled drugs, 7, 296
radioimaging agents, 18, 293
radioligand binding, 19, 283
radiosensitizers, 26, 151
ras GTPase, 26, 249
ras oncogene, 29, 165
receptor binding, 12, 249
receptor mapping, 14, 299; 15, 267; 23, 285
receptor modeling, 26, 281
receptor, concept and function, 21, 211
receptors, acetylcholine, 30, 41
receptors, adaptive changes, 19, 241
receptors, adenosine, 28, 295
receptors, adrenergic, 15, 217
receptors, ß-adrenergic blockers, 14, 81
receptors, benzodiazepine, 16, 21
receptors, cell surface, 12, 211
receptors, drug, 1, 236; 2, 227; 8, 262
receptors, G-protein coupled, 23, 221, 27, 291,
receptors, G-protein coupled CNS, 28, 29
receptors, histamine, 14, 91
receptors, muscarinic, 24, 31
receptors, neuropeptide, 28, 59
receptors, neuronal BZD, 28, 19

receptors, neurotransmitters, 3, 264; 12, 249
receptors, neuroleptic, 12, 249
receptors, opioid, 11, 33; 12, 20; 13, 41; 14, 31; 15, 32; 16, 41; 17, 21
receptors, peptide, 25, 281
receptors, serotonin, 23, 49
receptors, sigma, 28, 1
recombinant DNA, 17, 229; 18, 307; 19, 223
recombinant therapeutic proteins, 24, 213
renal blood flow, 16, 103
renin, 13, 82; 20, 257
reperfusion injury, 22, 253
reproduction, 1, 205; 2, 199; 3, 200; 4, 189
retinoids, 30, 119
reverse transcription, 8, 251
RGD-containing proteins, 28, 227
rheumatoid arthritis, 11, 138; 14, 219; 18, 171; 21, 201; 23, 171, 181
ribozymes, 30, 285
SAR, quantitative, 6, 245; 8, 313; 11, 301; 13, 292; 17, 291
sedative-hypnotics, 7, 39; 8, 29; 11, 13; 12, 10; 13, 21; 14, 22; 15, 22; 16, 31; 17, 11; 18, 11; 19, 11; 22, 11
sedatives, 1, 30; 2, 24; 3, 28; 4, 28; 7, 39; 8, 29; 10, 30; 11, 13; 12, 10; 13, 21; 14, 22; 15; 22; 16, 31; 17, 11; 18, 11; 20, 1; 21, 11
sequence-defined oligonucleotides, 26, 287
serotonergics, central, 25, 41; 27, 21
serotonin, 2, 273; 7, 47; 26, 103; 30, 1
serum lipoproteins, regulation, 13, 184
sexually-transmitted infections, 14, 114
SH2 domains, 30, 227
SH3 domains, 30, 227
silicon, in biology and medicine, 10, 265
sickle cell anemia, 20, 247
skeletal muscle relaxants, 8, 37
sleep, 27, 11
slow-reacting substances, 15, 69; 16, 213; 17, 203, 291
sodium/calcium exchange, 20, 215
solid state organic chemistry, 20, 287
solute active transport, 11, 222
somatostatin, 14, 209; 18, 199
spider toxins, 24, 287
SRS, 15, 69; 16, 213; 17, 203, 291
stereochemistry, Pharmaceutical, R&D, 25, 323
steroid hormones, 1, 213; 2, 208; 3, 207; 4, 199
stroidogenesis, adrenal, 2, 263
steroids, 2, 312; 3, 307; 4, 281; 5, 192, 296; 6, 162; 7, 182; 8, 194; 11, 192
stimulants, 1, 12; 2, 11; 3, 14; 4, 13; 5, 13; 6, 15; 7, 18; 8, 11
stroke, pharmacological approaches, 21, 108
stromelysin, biochemistry, 25, 177
structure-based drug design, 27, 271; 30, *265*
substance P, 17, 271; 18, 31
substituent constants, 2, 347
suicide enzyme inhibitors, 16, 289
superoxide dismutases, 10, 257
superoxide radical, 10, 257
sweeteners, non-nutritive, 17, 323
synthesis, asymmetric, 13, 282
synthesis, computer-assisted, 12, 288; 16, 281; 21, 203
synthesis, enzymic, 23, 305

T-cell antigen, 30, 199
T-cell receptors, 27, 189
tachykinins, 28, 99
taxol, 28, 305
thalidomide, 30, 319
thrombin, 30, 71
thrombolytic agents, 29, 93
thrombosis, 5, 237; 26, 93
thromboxane receptor antagonists, 25, 99
thromboxane synthase inhibitors, 25, 99
thromboxane synthetase, 22, 95
thromboxanes, 14, 178
thyrotropin releasing hormone, 17, 31
topoisomerase, 21, 247
toxicity reversal, 15, 233
toxicity, mathematical models, 18, 303
toxicology, comparative, 11, 242
toxins, bacterial, 12, 211
transcription factor NF-κB, 29, 235
transcription, reverse, 8, 251
transgenic animals, 24, 207
transgenic technology, 29, 265
translational control, 29, 245
traumatic injury, CNS, 25, 31
trophic factors, CNS, 27, 41
tumor necrosis factor, 22, 235
tyrosine kinase, 30, 247
vascular proliferative diseases, 30, 61
vasoactive peptides, 25, 89; 26, 83; 27, 79
vasoconstrictors, 4, 77
vasodilators, 4, 77; 12, 49
vasopressin antagonists, 23, 91
veterinary drugs, 16, 161
viruses, 14, 238
vitamin D, 10, 295; 15, 288; 17, 261; 19, 179
waking functions, 10, 21
water, structures, 5, 256
wound healing, 24, 223
xenobiotics, cyclic nucleotide metabolism, 15, 182
xenobiotic metabolism, 23, 315
x-ray crystallography, 21, 293; 27, 271

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
acarbose	antidiabetic	1990	26, 297
aceclofenac	antiinflammatory	1992	28, 325
acetohydroxamic acid	hypoammonuric	1983	19, 313
acetorphan	antidiarrheal	1993	29, 332
acipimox	hypolipidemic	1985	21, 323
acitretin	antipsoriatic	1989	25, 309
acrivastine	antihistamine	1988	24, 295
actarit	antirheumatic	1994	30, 296
adamantanium bromide	antiseptic	1984	20, 315
adrafinil	psychostimulant	1986	22, 315
AF-2259	antiinflammatory	1987	23, 325
afloqualone	muscle relaxant	1983	19, 313
alacepril	antihypertensive	1988	24, 296
alclometasone dipropionate	topical antiinflammatory	1985	21, 323
alendronate sodium	osteoporosis	1993	29, 332
alfentanil HCl	analgesic	1983	19, 314
alfuzosin HCl	antihypertensive	1988	24, 296
alglucerase	enzyme	1991	27, 321
alminoprofen	analgesic	1983	19, 314
alpha-1 antitrypsin	protease inhibitor	1988	24, 297
alpidem	anxiolytic	1991	27, 322
alpiropride	antimigraine	1988	24, 296
alteplase	thrombolytic	1987	23, 326
amfenac sodium	antiinflammatory	1986	22, 315
aminoprofen	topical antiinflammatory	1990	26, 298
amisulpride	antipsychotic	1986	22, 316
amlexanox	antiasthmatic	1987	23, 327
amlodipine besylate	antihypertensive	1990	26, 298
amorolfine hydrochloride	topical antifungal	1991	27, 322
amosulalol	antihypertensive	1988	24, 297
ampiroxicam	antiinflammatory	1994	30, 296
amrinone	cardiotonic	1983	19, 314
amsacrine	antineoplastic	1987	23, 327
amtolmetin guacil	antiinflammatory	1993	29, 332
angiotensin II	anticancer adjuvant	1994	30, 296
aniracetam	cognition enhancer	1993	29, 333
APD	calcium regulator	1987	23, 326
apraclonidine HCl	antiglaucoma	1988	24, 297
APSAC	thrombolytic	1987	23, 326
arbekacin	antibiotic	1990	26, 298
argatroban	antithromobotic	1990	26, 299
arotinolol HCl	antihypertensive	1986	22, 316
artemisinin	antimalarial	1987	23, 327
aspoxicillin	antibiotic	1987	23, 328
astemizole	antihistamine	1983	19, 314
astromycin sulfate	antibiotic	1985	21, 324
atovaquone	antiparasitic	1992	28, 326
auranofin	chrysotherapeutic	1983	19, 314
azelaic acid	antiacne	1989	25, 310
azelastine HCl	antihistamine	1986	22, 316
azithromycin	antibiotic	1988	24, 298
azosemide	diuretic	1986	22, 316
aztreonam	antibiotic	1984	20, 315
bambuterol	bronchodilator	1990	26, 299

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
barnidipine hydrochloride	antihypertensive	1992	28, 326
beclobrate	hypolipidemic	1986	22, 317
befunolol HCl	antiglaucoma	1983	19, 315
benazepril hydrochloride	antihypertensive	1990	26, 299
benexate HCl	antiulcer	1987	23, 328
benidipine hydrochloride	antihypertensive	1991	27, 322
beraprost sodium	platelet aggreg. inhibitor	1992	28, 326
betamethasone butyrate propionate	topical antiinflammatory	1994	30, 297
betaxolol HCl	antihypertensive	1983	19, 315
bevantolol HCl	antihypertensive	1987	23, 328
bifemelane HCl	nootropic	1987	23, 329
binfonazole	hypnotic	1983	19, 315
binifibrate	hypolipidemic	1986	22, 317
bisantrene hydrochloride	antineoplastic	1990	26, 300
bisoprolol fumarate	antihypertensive	1986	22, 317
bopindolol	antihypertensive	1985	21, 324
brodimoprin	antibiotic	1993	29, 333
brotizolam	hypnotic	1983	19, 315
brovincamine fumarate	cerebral vasodilator	1986	22, 317
bucillamine	immunomodulator	1987	23, 329
bucladesine sodium	cardiostimulant	1984	20, 316
budralazine	antihypertensive	1983	19, 315
bunazosin HCl	antihypertensive	1985	21, 324
bupropion HCl	antidepressant	1989	25, 310
buserelin acetate	hormone	1984	20, 316
buspirone HCl	anxiolytic	1985	21, 324
butenafine hydrochloride	topical antifungal	1992	28, 327
butibufen	antiinflammatory	1992	28, 327
butoconazole	topical antifungal	1986	22, 318
butoctamide	hypnotic	1984	20, 316
butyl flufenamate	topical antiinflammatory	1983	19, 316
cabergoline	antiprolactin	1993	29, 334
cadexomer iodine	wound healing agent	1983	19, 316
cadralazine	hypertensive	1988	24, 298
calcipotriol	antipsoriatic	1991	27, 323
camostat mesylate	antineoplastic	1985	21, 325
carboplatin	antibiotic	1986	22, 318
carumonam	antibiotic	1988	24, 298
carvedilol	antihypertensive	1991	27, 323
cefbuperazone sodium	antibiotic	1985	21, 325
cefdinir	antibiotic	1991	27, 323
cefditoren pivoxil	oral cephalosporin	1994	30, 297
cefepime	antibiotic	1993	29, 334
cefetamet pivoxil hydrochloride	antibiotic	1992	28, 327
cefixime	antibiotic	1987	23, 329
cefmenoxime HCl	antibiotic	1983	19, 316
cefminox sodium	antibiotic	1987	23, 330
cefodizime sodium	antibiotic	1990	26, 300
cefonicid sodium	antibiotic	1984	20, 316
ceforanide	antibiotic	1984	20, 317
cefotetan disodium	antibiotic	1984	20, 317

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cefotiam hexetil hydrochloride	antibiotic	1991	27, 324
cefpimizole	antibiotic	1987	23, 330
cefpiramide sodium	antibiotic	1985	21, 325
cefpirome sulfate	antibiotic	1992	28, 328
cefpodoxime proxetil	antibiotic	1989	25, 310
cefprozil	antibiotic	1992	28, 328
ceftazidime	antibiotic	1983	19, 316
cefteram pivoxil	antibiotic	1987	23, 330
ceftibuten	antibiotic	1992	28, 329
cefuroxime axetil	antibiotic	1987	23, 331
cefuzonam sodium	antibiotic	1987	23, 331
celiprolol HCl	antihypertensive	1983	19, 317
centchroman	antiestrogen	1991	27, 324
centoxin	immunomodulator	1991	27, 325
cetirizine HCl	antihistamine	1987	23, 331
chenodiol	anticholelithogenic	1983	19, 317
choline alfoscerate	nootropic	1990	26, 300
cibenzoline	antiarrhythmic	1985	21, 325
cicletanine	antihypertensive	1988	24, 299
cilazapril	antihypertensive	1990	26, 301
cilostazol	antithrombotic	1988	24, 299
cimetropium bromide	antispasmodic	1985	21, 326
cinitapride	gastroprokinetic	1990	26, 301
cinolazepam	hypnotic	1993	29, 334
ciprofibrate	hypolipidemic	1985	21, 326
ciprofloxacin	antibacterial	1986	22, 318
cisapride	gastroprokinetic	1988	24, 299
citalopram	antidepressant	1989	25, 311
cladribine	antineoplastic	1993	29, 335
clarithromycin	antibiotic	1990	26, 302
clobenoside	vasoprotective	1988	24, 300
cloconazole HCl	topical antifungal	1986	22, 318
clodronate disodium	calcium regulator	1986	22, 319
cloricromen	antithrombotic	1991	27, 325
clospipramine hydrochloride	neuroleptic	1991	27, 325
cyclosporine	immunosuppressant	1983	19, 317
cytarabine ocfosfate	antineoplastic	1993	29, 335
dapiprazole HCl	antiglaucoma	1987	23, 332
defibrotide	antithrombotic	1986	22, 319
deflazacort	antiinflammatory	1986	22, 319
delapril	antihypertensive	1989	25, 311
denopamine	cardiostimulant	1988	24, 300
deprodone propionate	topical antiinflammatory	1992	28, 329
desflurane	anesthetic	1992	28, 329
dexibuprofen	antiinflammatory	1994	30, 298
dexrazoxane	cardioprotective	1992	28, 330
dezocine	analgesic	1991	27, 326
diacerein	antirheumatic	1985	21, 326
didanosine	antiviral	1991	27, 326
dilevalol	antihypertensive	1989	25, 311
dirithromycin	antibiotic	1993	29, 336
disodium pamidronate	calcium regulator	1989	25, 312
divistyramine	hypocholesterolemic	1984	20, 317

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
docarpamine	cardiostimulant	1994	30, 298
dopexamine	cardiostimulant	1989	25, 312
dornase alfa	cystic fibrosis	1994	30, 298
doxacurium chloride	muscle relaxant	1991	27, 326
doxazosin mesylate	antihypertensive	1988	24, 300
doxefazepam	hypnotic	1985	21, 326
doxifluridine	antineoplastic	1987	23, 332
doxofylline	bronchodilator	1985	21, 327
dronabinol	antinauseant	1986	22, 319
droxicam	antiinflammatory	1990	26, 302
droxidopa	antiparkinsonian	1989	25, 312
ebastine	antihistamine	1990	26 302
ecabet sodium	antiulcerative	1993	29, 336
efonidipine	antihypertensive	1994	30, 299
emedastine difumarate	antiallergic/antiasthmatic	1993	29, 336
emorfazone	analgesic	1984	20, 317
enalapril maleate	antihypertensive	1984	20, 317
enalaprilat	antihypertensive	1987	23, 332
encainide HCl	antiarrhythmic	1987	23, 333
enocitabine	antineoplastic	1983	19, 318
enoxacin	antibacterial	1986	22, 320
enoxaparin	antithrombotic	1987	23, 333
enoximone	cardiostimulant	1988	24, 301
enprostil	antiulcer	1985	21, 327
epalrestat	antidiabetic	1992	28, 330
eperisone HCl	muscle relaxant	1983	19, 318
epidermal growth factor	wound healing agent	1987	23, 333
epinastine	antiallergic	1994	30, 299
epirubicin HCl	antineoplastic	1984	20, 318
epoprostenol sodium	platelet aggreg. inhib.	1983	19, 318
eptazocine HBr	analgesic	1987	23, 334
erythromycin acistrate	antibiotic	1988	24, 301
erythropoietin	hematopoetic	1988	24, 301
esmolol HCl	antiarrhythmic	1987	23, 334
ethyl icosapentate	antithrombotic	1990	26, 303
etizolam	anxiolytic	1984	20, 318
etodolac	antiinflammatory	1985	21, 327
exifone	nootropic	1988	24, 302
factor VIII	hemostatic	1992	28, 330
famciclovir	antiviral	1994	30, 300
famotidine	antiulcer	1985	21, 327
felbamate	antiepileptic	1993	29, 337
felbinac	topical antiinflammatory	1986	22, 320
felodipine	antihypertensive	1988	24, 302
fenbuprol	choleretic	1983	19, 318
fenticonazole nitrate	antifungal	1987	23, 334
filgrastim	immunostimulant	1991	27, 327
finasteride	5α-reductase inhibitor	1992	28, 331
fisalamine	intestinal antiinflammatory	1984	20, 318
fleroxacin	antibacterial	1992	28, 331
flomoxef sodium	antibiotic	1988	24, 302
flosequinan	cardiostimulant	1992	28, 331
fluconazole	antifungal	1988	24, 303

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
fludarabine phosphate	antineoplastic	1991	27, 327
flumazenil	benzodiazepine antag.	1987	23, 335
flunoxaprofen	antiinflammatory	1987	23, 335
fluoxetine HCl	antidepressant	1986	22, 320
flupirtine maleate	analgesic	1985	21, 328
flutamide	antineoplastic	1983	19, 318
flutazolam	anxiolytic	1984	20, 318
fluticasone propionate	antiinflammatory	1990	26, 303
flutoprazepam	anxiolytic	1986	22, 320
flutropium bromide	antitussive	1988	24, 303
fluvastatin	hypolipaemic	1994	30, 300
fluvoxamine maleate	antidepressant	1983	19, 319
formestane	antineoplastic	1993	29, 337
formoterol fumarate	bronchodilator	1986	22, 321
foscarnet sodium	antiviral	1989	25, 313
fosfosal	analgesic	1984	20, 319
fosinopril sodium	antihypertensive	1991	27, 328
fotemustine	antineoplastic	1989	25, 313
gabapentin	antiepileptic	1993	29, 338
gallium nitrate	calcium regulator	1991	27, 328
gallopamil HCl	antianginal	1983	19, 319
ganciclovir	antiviral	1988	24, 303
gemeprost	abortifacient	1983	19, 319
gestodene	progestogen	1987	23, 335
gestrinone	antiprogestogen	1986	22, 321
glucagon, rDNA	hypoglycemia	1993	29, 338
goserelin	hormone	1987	23, 336
granisetron hydrochloride	antiemetic	1991	27, 329
guanadrel sulfate	antihypertensive	1983	19, 319
gusperimus	immunosuppressant	1994	30, 300
halobetasol propionate	topical antiinflammatory	1991	27, 329
halofantrine	antimalarial	1988	24, 304
halometasone	topical antiinflammatory	1983	19, 320
histrelin	precocious puberty	1993	29, 338
hydrocortisone aceponate	topical antiinflammatory	1988	24, 304
hydrocortisone butyrate	topical antiinflammatory	1983	19, 320
ibopamine HCl	cardiostimulant	1984	20, 319
ibudilast	antiasthmatic	1989	25, 313
idarubicin hydrochloride	antineoplastic	1990	26, 303
idebenone	nootropic	1986	22, 321
iloprost	platelet aggreg. inhibitor	1992	28, 332
imidapril HCl	antihypertensive	1993	29, 339
imiglucerase	Gaucher's disease	1994	30, 301
imipenem/cilastatin	antibiotic	1985	21, 328
indalpine	antidepressant	1983	19, 320
indeloxazine HCl	nootropic	1988	24, 304
indobufen	antithrombotic	1984	20, 319
interferon, β-1b	multiple sclerosis	1993	29, 339
interferon, gamma	antiinflammatory	1989	25, 314
interferon, gamma-1α	antineoplastic	1992	28, 332
interferon gamma-1b	immunostimulant	1991	27, 329
interleukin-2	antineoplastic	1989	25, 314
ipriflavone	calcium regulator	1989	25, 314

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
irinotecan	antineoplastic	1994	30, 301
irsogladine	antiulcer	1989	25, 315
isepamicin	antibiotic	1988	24, 305
isofezolac	antiinflammatory	1984	20, 319
isoxicam	antiinflammatory	1983	19, 320
isradipine	antihypertensive	1989	25, 315
itraconazole	antifungal	1988	24, 305
ivermectin	antiparasitic	1987	23, 336
ketanserin	antihypertensive	1985	21, 328
ketorolac tromethamine	analgesic	1990	26, 304
lacidipine	antihypertensive	1991	27, 330
lamotrigine	anticonvulsant	1990	26, 304
lanoconazole	antifungal	1994	30, 302
lansoprazole	antiulcer	1992	28, 332
lenampicillin HCl	antibiotic	1987	23, 336
lentinan	immunostimulant	1986	22, 322
leuprolide acetate	hormone	1984	20, 319
levacecarnine HCl	nootropic	1986	22, 322
levobunolol HCl	antiglaucoma	1985	21, 328
levocabastine hydrochloride	antihistamine	1991	27, 330
levodropropizine	antitussive	1988	24, 305
levofloxacin	antibiotic	1993	29, 340
lidamidine HCl	antiperistaltic	1984	20, 320
limaprost	antithrombotic	1988	24, 306
lisinopril	antihypertensive	1987	23, 337
lobenzarit sodium	antiinflammatory	1986	22, 322
lodoxamide tromethamine	antiallergic ophthalmic	1992	28, 333
lomefloxacin	antibiotic	1989	25, 315
lonidamine	antineoplastic	1987	23, 337
loprazolam mesylate	hypnotic	1983	19, 321
loracarbef	antibiotic	1992	28, 333
loratadine	antihistamine	1988	24, 306
losartan	antihypertensive	1994	30, 302
lovastatin	hypocholesterolemic	1987	23, 337
loxoprofen sodium	antiinflammatory	1986	22, 322
mabuterol HCl	bronchodilator	1986	22, 323
malotilate	hepatroprotective	1985	21, 329
manidipine hydrochloride	antihypertensive	1990	26, 304
masoprocol	topical antineoplastic	1992	28, 333
medifoxamine fumarate	antidepressant	1986	22, 323
mefloquine HCl	antimalarial	1985	21, 329
meglutol	hypolipidemic	1983	19, 321
melinamide	hypocholesterolemic	1984	20, 320
mepixanox	analeptic	1984	20, 320
meptazinol HCl	analgesic	1983	19, 321
meropenem	carbapenem antibiotic	1994	30, 303
metaclazepam	anxiolytic	1987	23, 338
metapramine	antidepressant	1984	20, 320
mexazolam	anxiolytic	1984	20, 321
mifepristone	abortifacient	1988	24, 306
milrinone	cardiostimulant	1989	25, 316
miltefosine	topical antineoplastic	1993	29, 340
miokamycin	antibiotic	1985	21, 329
mirtazapine	antidepressant	1994	30, 303

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
misoprostol	antiulcer	1985	21, 329
mivacurium chloride	muscle relaxant	1992	28, 334
mitoxantrone HCl	antineoplastic	1984	20, 321
mizoribine	immunosuppressant	1984	20, 321
moclobemide	antidepressant	1990	26, 305
modafinil	idiopathic hypersomnia	1994	30, 303
mofezolac	analgesic	1994	30, 304
mometasone furoate	topical antiinflammatory	1987	23, 338
moricizine hydrochloride	antiarrhythmic	1990	26, 305
moxonidine	antihypertensive	1991	27, 330
mupirocin	topical antibiotic	1985	21, 330
muromonab-CD3	immunosuppressant	1986	22, 323
muzolimine	diuretic	1983	19, 321
nabumetone	antiinflammatory	1985	21, 330
nadifloxacin	topical antibiotic	1993	29, 340
nafamostat mesylate	protease inhibitor	1986	22, 323
nafarelin acetate	hormone	1990	26, 306
naftifine HCl	antifungal	1984	20, 321
naltrexone HCl	narcotic antagonist	1984	20, 322
nartograstim	leukopenia	1994	30, 304
nazasetron	antiemetic	1994	30, 305
nedocromil sodium	antiallergic	1986	22, 324
nefazodone	antidepressant	1994	30, 305
neltenexine	cystic fibrosis	1993	29, 341
nemonapride	neuroleptic	1991	27, 331
neticonazole HCl	topical antifungal	1993	29, 341
nicorandil	coronary vasodilator	1984	20, 322
nilutamide	antineoplastic	1987	23, 338
nilvadipine	antihypertensive	1989	25, 316
nimesulide	antiinflammatory	1985	21, 330
nimodipine	cerebral vasodilator	1985	21, 330
nipradilol	antihypertensive	1988	24, 307
nisoldipine	antihypertensive	1990	26, 306
nitrefazole	alcohol deterrent	1983	19, 322
nitrendipine	hypertensive	1985	21, 331
nizatidine	antiulcer	1987	23, 339
nizofenzone fumarate	nootropic	1988	24, 307
nomegestrol acetate	progestogen	1986	22, 324
norfloxacin	antibacterial	1983	19, 322
norgestimate	progestogen	1986	22, 324
octreotide	antisecretory	1988	24, 307
ofloxacin	antibacterial	1985	21, 331
omeprazole	antiulcer	1988	24, 308
ondansetron hydrochloride	antiemetic	1990	26, 306
ornoprostil	antiulcer	1987	23, 339
osalazine sodium	intestinal antinflamm.	1986	22, 324
oxaprozin	antiinflammatory	1983	19, 322
oxcarbazepine	anticonvulsant	1990	26, 307
oxiconazole nitrate	antifungal	1983	19, 322
oxiracetam	nootropic	1987	23, 339
oxitropium bromide	bronchodilator	1983	19, 323
ozagrel sodium	antithrombotic	1988	24, 308
paclitaxal	antineoplastic	1993	29, 342
parnaparin sodium	anticoagulant	1993	29, 342

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
panipenem/betamipron	carbapenem antibiotic	1994	30, 305
pantoprazole sodium	antiulcer	1995	30, 306
paroxetine	antidepressant	1991	27, 331
pefloxacin mesylate	antibacterial	1985	21, 331
pegademase bovine	immunostimulant	1990	26, 307
pegaspargase	antineoplastic	1994	30, 306
pemirolast potassium	antiasthmatic	1991	27, 331
pentostatin	antineoplastic	1992	28, 334
pergolide mesylate	antiparkinsonian	1988	24, 308
perindopril	antihypertensive	1988	24, 309
picotamide	antithrombotic	1987	23, 340
pidotimod	immunostimulant	1993	29, 343
piketoprofen	topical antiinflammatory	1984	20, 322
pilsicainide hydrochloride	antiarrhythmic	1991	27, 332
pimaprofen	topical antiinflammatory	1984	20, 322
pimobendan	heart failure	1994	30, 307
pinacidil	antihypertensive	1987	23, 340
pirarubicin	antineoplastic	1988	24, 309
pirmenol	antiarrhythmic	1994	30, 307
piroxicam cinnamate	antiinflammatory	1988	24, 309
plaunotol	antiulcer	1987	23, 340
polaprezinc	antiulcer	1994	30, 307
porfimer sodium	antineoplastic adjuvant	1993	29, 343
pramiracetam H_2SO_4	cognition enhancer	1993	29, 343
pravastatin	antilipidemic	1989	25, 316
prednicarbate	topical antiinflammatory	1986	22, 325
progabide	anticonvulsant	1985	21, 331
promegestrone	progestogen	1983	19, 323
propacetamol HCl	analgesic	1986	22, 325
propagermanium	antiviral	1994	30, 308
propentofylline propionate	cerebral vasodilator	1988	24, 310
propiverine hydrochloride	urologic	1992	28, 335
propofol	anesthetic	1986	22, 325
pumactant	lung surfactant	1994	30, 308
quazepam	hypnotic	1985	21, 332
quinagolide	hyperprolactinemia	1994	30, 309
quinapril	antihypertensive	1989	25, 317
quinfamide	amebicide	1984	20, 322
ramipril	antihypertensive	1989	25, 317
ranimustine	antineoplastic	1987	23, 341
rebamipide	antiulcer	1990	26, 308
remoxipride hydrochloride	antipsychotic	1990	26, 308
repirinast	antiallergic	1987	23, 341
reviparin sodium	anticoagulant	1993	29, 344
rifabutin	antibacterial	1992	28, 335
rifapentine	antibacterial	1988	24, 310
rifaximin	antibiotic	1985	21, 332
rifaximin	antibiotic	1987	23, 341
rilmazafone	hypnotic	1989	25, 317
rilmenidine	antihypertensive	1988	24, 310
rimantadine HCl	antiviral	1987	23, 342
risperidone	neuroleptic	1993	29, 344
rocuronium bromide	neuromuscular blocker	1994	30, 309
rokitamycin	antibiotic	1986	22, 325

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
romurtide	immunostimulant	1991	27, 332
ronafibrate	hypolipidemic	1986	22, 326
rosaprostol	antiulcer	1985	21, 332
roxatidine acetate HCl	antiulcer	1986	22, 326
roxithromycin	antiulcer	1987	23, 342
rufloxacin hydrochloride	antibacterial	1992	28, 335
RV-11	antibiotic	1989	25, 318
salmeterol hydroxynaphthoate	bronchodilator	1990	26, 308
sapropterin hydrochloride	hyperphenylalaninemia	1992	28, 336
sargramostim	immunostimulant	1991	27, 332
sarpogrelate HCl	platelet antiaggregant	1993	29, 344
schizophyllan	immunostimulant	1985	22, 326
sertaconazole nitrate	topical antifungal	1992	28, 336
setastine HCl	antihistamine	1987	23, 342
setiptiline	antidepressant	1989	25, 318
setraline hydrochloride	antidepressant	1990	26, 309
sevoflurane	anesthetic	1990	26, 309
simvastatin	hypocholesterolemic	1988	24, 311
sobuzoxane	antineoplastic	1994	30, 310
sodium cellulose PO4	hypocalciuric	1983	19, 323
sofalcone	antiulcer	1984	20, 323
somatomedin-1	growth hormone insensitivity	1994	30, 310
somatotropin	growth hormone	1994	30, 310
somatropin	hormone	1987	23, 343
sorivudine	antiviral	1993	29, 345
sparfloxacin	antibiotic	1993	29, 345
spizofurone	antiulcer	1987	23, 343
stavudine	antiviral	1994	30, 311
succimer	chelator	1991	27, 333
sufentanil	analgesic	1983	19, 323
sulbactam sodium	B-lactamase inhibitor	1986	22, 326
sulconizole nitrate	topical antifungal	1985	21, 332
sultamycillin tosylate	antibiotic	1987	23, 343
sumatriptan succinate	antimigraine	1991	27, 333
suprofen	analgesic	1983	19, 324
surfactant TA	respiratory surfactant	1987	23, 344
tacalcitol	topical antipsoriatic	1993	29, 346
tacrine HCl	Alzheimer's disease	1993	29, 346
tacrolimus	immunosuppressant	1993	29, 347
tamsulosin HCl	antiprostatic hypertrophy	1993	29, 347
tazobactam sodium	β-lactamase inhibitor	1992	28, 336
tazanolast	antiallergic	1990	26, 309
teicoplanin	antibacterial	1988	24, 311
telmesteine	mucolytic	1992	28, 337
temafloxacin hydrochloride	antibacterial	1991	27, 334
temocapril	antihypertensive	1994	30, 311
temocillin disodium	antibiotic	1984	20, 323
tenoxicam	antiinflammatory	1987	23, 344
teprenone	antiulcer	1984	20, 323
terazosin HCl	antihypertensive	1984	20, 323
terbinafine hydrochloride	antifungal	1991	27, 334

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
terconazole	antifungal	1983	19, 324
tertatolol HCl	antihypertensive	1987	23, 344
thymopentin	immunomodulator	1985	21, 333
tiamenidine HCl	antihypertensive	1988	24, 311
tianeptine sodium	antidepressant	1983	19, 324
tibolone	anabolic	1988	24, 312
tilisolol hydrochloride	antihypertensive	1992	28, 337
timiperone	neuroleptic	1984	20, 323
tinazoline	nasal decongestant	1988	24, 312
tioconazole	antifungal	1983	19, 324
tiopronin	urolithiasis	1989	25, 318
tiquizium bromide	antispasmodic	1984	20, 324
tiracizine hydrochloride	antiarrhythmic	1990	26, 310
tiropramide HCl	antispasmodic	1983	19, 324
tizanidine	muscle relaxant	1984	20, 324
toloxatone	antidepressant	1984	20, 324
tolrestat	antidiabetic	1989	25, 319
torasemide	diuretic	1993	29, 348
toremifene	antineoplastic	1989	25, 319
tosufloxacin tosylate	antibacterial	1990	26, 310
trandolapril	antihypertensive	1993	29, 348
tretinoin tocoferil	antiulcer	1993	29, 348
trientine HCl	chelator	1986	22, 327
trimazosin HCl	antihypertensive	1985	21, 333
trimetrexate glucuronate	*Pneumocystis carinii* pneumonia	1994	30, 312
tropisetron	antiemetic	1992	28, 337
troxipide	antiulcer	1986	22, 327
ubenimex	immunostimulant	1987	23, 345
unoprostone isopropyl ester	antiglaucoma	1994	30, 312
venlafaxine	antidepressant	1994	30, 312
vesnarinone	cardiostimulant	1990	26, 310
vigabatrin	anticonvulsant	1989	25, 319
vinorelbine	antineoplastic	1989	25, 320
voglibose	antidiabetic	1994	30, 313
xamoterol fumarate	cardiotonic	1988	24, 312
zalcitabine	antiviral	1992	28, 338
zaltoprofen	antiinflammatory	1993	29, 349
zidovudine	antiviral	1987	23, 345
zinostatin stimalamer	antineoplastic	1994	30, 313
zolpidem hemitartrate	hypnotic	1988	24, 313
zonisamide	anticonvulsant	1989	25, 320
zopiclone	hypnotic	1986	22, 327
zuclopenthixol acetate	antipsychotic	1987	23, 345

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
gemeprost	ABORTIFACIENT	1983	19,319
mifepristone		1988	24,306
nitrefazole	ALCOHOL DETERRENT	1983	19,322
tacrine HCl	ALZHEIMER'S DISEASE	1993	29,346
quinfamide	AMEBICIDE	1984	20,322
tibolone	ANABOLIC	1988	24,312
mepixanox	ANALEPTIC	1984	20,320
alfentanil HCl	ANALGESIC	1983	19,314
alminoprofen		1983	19,314
dezocine		1991	27,326
emorfazone		1984	20,317
eptazocine HBr		1987	23,334
flupirtine maleate		1985	21,328
fosfosal		1984	20,319
ketorolac tromethamine		1990	26,304
meptazinol HCl		1983	19,321
mofezolac		1994	30,304
propacetamol HCl		1986	22,325
sufentanil		1983	19,323
suprofen		1983	19,324
desflurane	ANESTHETIC	1992	28,329
propofol		1986	22,325
sevoflurane		1990	26,309
azelaic acid	ANTIACNE	1989	25,310
emedastine difumarate	ANTIALLERGIC	1993	29,336
epinastine		1994	30,299
nedocromil sodium		1986	22,324
repirinast		1987	23,341
tazanolast		1990	26,309
lodoxamide tromethamine	ANTIALLERGIC OPHTHALMIC	1992	28,333
gallopamil HCl	ANTIANGINAL	1983	19,319
cibenzoline	ANTIARRHYTHMIC	1985	21,325
encainide HCl		1987	23,333
esmolol HCl		1987	23,334
moricizine hydrochloride		1990	26,305
pilsicainide hydrochloride		1991	27,332
pirmenol		1994	30,307
tiracizine hydrochloride		1990	26,310
amlexanox	ANTIASTHMATIC	1987	23,327
emedastine difumarate		1993	29,336

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
ibudilast		1989	25,313
pemirolast potassium		1991	27,331
ciprofloxacin	ANTIBACTERIAL	1986	22,318
enoxacin		1986	22,320
fleroxacin		1992	28,331
norfloxacin		1983	19,322
ofloxacin		1985	21,331
pefloxacin mesylate		1985	21,331
rifabutin		1992	28,335
rifapentine		1988	24,310
rufloxacin hydrochloride		1992	28,335
teicoplanin		1988	24,311
temafloxacin hydrochloride		1991	27,334
tosufloxacin tosylate		1990	26,310
arbekacin	ANTIBIOTIC	1990	26,298
aspoxicillin		1987	23,328
astromycin sulfate		1985	21,324
azithromycin		1988	24,298
aztreonam		1984	20,315
brodimoprin		1993	29,333
carboplatin		1986	22,318
carumonam		1988	24,298
cefbuperazone sodium		1985	21,325
cefdinir		1991	27,323
cefepime		1993	29,334
cefetamet pivoxil hydrochloride		1992	28,327
cefixime		1987	23,329
cefmenoxime HCl		1983	19,316
cefminox sodium		1987	23,330
cefodizime sodium		1990	26,300
cefonicid sodium		1984	20,316
ceforanide		1984	20,317
cefotetan disodium		1984	20,317
cefotiam hexetil hydrochloride		1991	27,324
cefpimizole		1987	23,330
cefpiramide sodium		1985	21,325
cefpirome sulfate		1992	28,328
cefpodoxime proxetil		1989	25,310
cefprozil		1992	28,328
ceftazidime		1983	19,316
cefteram pivoxil		1987	23,330
ceftibuten		1992	28,329
cefuroxime axetil		1987	23,331
cefuzonam sodium		1987	23,331
clarithromycin		1990	26,302
dirithromycin		1993	29,336
erythromycin acistrate		1988	24,301
flomoxef sodium		1988	24,302
imipenem/cilastatin		1985	21,328
isepamicin		1988	24,305
lenampicillin HCl		1987	23,336
levofloxacin		1993	29,340

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
lomefloxacin		1989	25,315
loracarbef		1992	28,333
miokamycin		1985	21,329
rifaximin		1985	21,332
rifaximin		1987	23,341
rokitamycin		1986	22,325
RV-11		1989	25,318
sparfloxacin		1993	29,345
sultamycillin tosylate		1987	23,343
temocillin disodium		1984	20,323
meropenem	ANTIBIOTIC, CARBAPENEM	1994	30,303
panipenem/betamipron		1994	30,305
mupirocin	ANTIBIOTIC, TOPICAL	1985	21,330
nadifloxacin		1993	29,340
angiotensin II	ANTICANCER ADJUVANT	1994	30,296
chenodiol	ANTICHOLELITHOGENIC	1983	19,317
parnaparin sodium	ANTICOAGULANT	1993	29,342
reviparin sodium		1993	29,344
lamotrigine	ANTICONVULSANT	1990	26,304
oxcarbazepine		1990	26,307
progabide		1985	21,331
vigabatrin		1989	25,319
zonisamide		1989	25,320
bupropion HCl	ANTIDEPRESSANT	1989	25,310
citalopram		1989	25,311
fluoxetine HCl		1986	22,320
fluvoxamine maleate		1983	19,319
indalpine		1983	19,320
medifoxamine fumarate		1986	22,323
metapramine		1984	20,320
mirtazapine		1994	30,303
moclobemide		1990	26,305
nefazodone		1994	30,305
paroxetine		1991	27,331
setiptiline		1989	25,318
sertraline hydrochloride		1990	26,309
tianeptine sodium		1983	19,324
toloxatone		1984	20,324
venlafaxine		1994	30,312
acarbose	ANTIDIABETIC	1990	26,297
epalrestat		1992	28,330
tolrestat		1989	25,319
voglibose		1994	30,313
acetorphan	ANTIDIARRHEAL	1993	29,332

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
granisetron hydrochloride	ANTIEMETIC	1991	27,329
ondansetron hydrochloride		1990	26,306
nazasetron		1994	30,305
tropisetron		1992	28,337
felbamate	ANTIEPILEPTIC	1993	29,337
gabapentin		1993	29,338
centchroman	ANTIESTROGEN	1991	27,324
fenticonazole nitrate	ANTIFUNGAL	1987	23,334
fluconazole		1988	24,303
itraconazole		1988	24,305
lanoconazole		1994	30,302
naftifine HCl		1984	20,321
oxiconazole nitrate		1983	19,322
terbinafine hydrochloride		1991	27,334
terconazole		1983	19,324
tioconazole		1983	19,324
amorolfine hydrochloride	ANTIFUNGAL, TOPICAL	1991	27,322
butenafine hydrochloride		1992	28,327
butoconazole		1986	22,318
cloconazole HCl		1986	22,318
neticonazole HCl		1993	29,341
sertaconazole nitrate		1992	28,336
sulconizole nitrate		1985	21,332
apraclonidine HCl	ANTIGLAUCOMA	1988	24,297
befunolol HCl		1983	19,315
dapiprazole HCl		1987	23,332
levobunolol HCl		1985	21,328
unoprostone isopropyl ester		1994	30,312
acrivastine	ANTIHISTAMINE	1988	24,295
astemizole		1983	19,314
azelastine HCl		1986	22,316
ebastine		1990	26,302
cetirizine HCl		1987	23,331
levocabastine hydrochloride		1991	27,330
loratadine		1988	24,306
setastine HCl		1987	23,342
alacepril	ANTIHYPERTENSIVE	1988	24,296
alfuzosin HCl		1988	24,296
amlodipine besylate		1990	26,298
amosulalol		1988	24,297
arotinolol HCl		1986	22,316
barnidipine hydrochloride		1992	28,326
benazepril hydrochloride		1990	26,299
benidipine hydrochloride		1991	27,322
betaxolol HCl		1983	19,315
bevantolol HCl		1987	23,328
bisoprolol fumarate		1986	22,317

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
bopindolol		1985	21,324
budralazine		1983	19,315
bunazosin HCl		1985	21,324
carvedilol		1991	27,323
celiprolol HCl		1983	19,317
cicletanine		1988	24,299
cilazapril		1990	26,301
delapril		1989	25,311
dilevalol		1989	25,311
doxazosin mesylate		1988	24,300
efonidipine		1994	30,299
enalapril maleate		1984	20,317
enalaprilat		1987	23,332
felodipine		1988	24,302
fosinopril sodium		1991	27,328
guanadrel sulfate		1983	19,319
imidapril HCl		1993	29,339
isradipine		1989	25,315
ketanserin		1985	21,328
lacidipine		1991	27,330
lisinopril		1987	23,337
losartan		1994	30,302
manidipine hydrochloride		1990	26,304
moxonidine		1991	27,330
nilvadipine		1989	25,316
nipradilol		1988	24,307
nisoldipine		1990	26,306
perindopril		1988	24,309
pinacidil		1987	23,340
quinapril		1989	25,317
ramipril		1989	25,317
rilmenidine		1988	24,310
temocapril		1994	30,311
terazosin HCl		1984	20,323
tertatolol HCl		1987	23,344
tiamenidine HCl		1988	24,311
tilisolol hydrochloride		1992	28,337
trandolapril		1993	29,348
trimazosin HCl		1985	21,333
aceclofenac	ANTIINFLAMMATORY	1992	28,325
AF-2259		1987	23,325
amfenac sodium		1986	22,315
ampiroxicam		1994	30,296
amtolmetin guacil		1993	29,332
butibufen		1992	28,327
deflazacort		1986	22,319
dexibuprofen		1994	30,298
droxicam		1990	26,302
etodolac		1985	21,327
flunoxaprofen		1987	23,335
fluticasone propionate		1990	26,303
interferon, gamma		1989	25,314
isofezolac		1984	20,319

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
isoxicam		1983	19,320
lobenzarit sodium		1986	22,322
loxoprofen sodium		1986	22,322
nabumetone		1985	21,330
nimesulide		1985	21,330
oxaprozin		1983	19,322
piroxicam cinnamate		1988	24,309
tenoxicam		1987	23,344
zaltoprofen		1993	29,349
fisalamine	ANTIINFLAMMATORY, INTESTINAL	1984	20,318
osalazine sodium		1986	22,324
alclometasone dipropionate	ANTIINFLAMMATORY, TOPICAL	1985	21,323
aminoprofen		1990	26,298
betamethasone butyrate propionate		1994	30,297
butyl flufenamate		1983	19,316
deprodone propionate		1992	28,329
felbinac		1986	22,320
halobetasol propionate		1991	27,329
halometasone		1983	19,320
hydrocortisone aceponate		1988	24,304
hydrocortisone butyrate propionate		1983	19,320
mometasone furoate		1987	23,338
piketoprofen		1984	20,322
pimaprofen		1984	20,322
prednicarbate		1986	22,325
pravastatin	ANTILIPIDEMIC	1989	25,316
artemisinin	ANTIMALARIAL	1987	23,327
halofantrine		1988	24,304
mefloquine HCl		1985	21,329
alpiropride	ANTIMIGRAINE	1988	24,296
sumatriptan succinate		1991	27,333
dronabinol	ANTINAUSEANT	1986	22,319
amsacrine	ANTINEOPLASTIC	1987	23,327
bisantrene hydrochloride		1990	26,300
camostat mesylate		1985	21,325
cladribine		1993	29,335
cytarabine ocfosfate		1993	29,335
doxifluridine		1987	23,332
enocitabine		1983	19,318
epirubicin HCl		1984	20,318
fludarabine phosphate		1991	27,327
flutamide		1983	19,318
formestane		1993	29,337
fotemustine		1989	25,313
idarubicin hydrochloride		1990	26,303

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
interferon gamma-1 α		1992	28,332
interleukin-2		1989	25,314
irinotecan		1994	30,301
lonidamine		1987	23,337
mitoxantrone HCl		1984	20,321
nilutamide		1987	23,338
paclitaxal		1993	29,342
pegaspargase		1994	30,306
pentostatin		1992	28,334
pirarubicin		1988	24,309
ranimustine		1987	23,341
sobuzoxane		1994	30,310
toremifene		1989	25,319
vinorelbine		1989	25,320
zinostatin stimalamer		1994	30,313
porfimer sodium	ANTINEOPLASTIC ADJUVANT	1993	29,343
masoprocol	ANTINEOPLASTIC, TOPICAL	1992	28,333
miltefosine		1993	29,340
atovaquone	ANTIPARASITIC	1992	28,326
ivermectin		1987	23,336
droxidopa	ANTIPARKINSONIAN	1989	25,312
pergolide mesylate		1988	24,308
lidamidine HCl	ANTIPERISTALTIC	1984	20,320
gestrinone	ANTIPROGESTOGEN	1986	22,321
cabergoline	ANTIPROLACTIN	1993	29,334
tamsulosin HCl	ANTIPROSTATIC HYPERTROPHY	1993	29,347
acitretin	ANTIPSORIATIC	1989	25,309
calcipotriol		1991	27,323
tacalcitol	ANTIPSORIATIC, TOPICAL	1993	29,346
amisulpride	ANTIPSYCHOTIC	1986	22,316
remoxipride hydrochloride		1990	26,308
zuclopenthixol acetate		1987	23,345
actarit	ANTIRHEUMATIC	1994	30,296
diacerein		1985	21,326
octreotide	ANTISECRETORY	1988	24,307
adamantanium bromide	ANTISEPTIC	1984	20,315

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
cimetropium bromide	ANTISPASMODIC	1985	21,326
tiquizium bromide		1984	20,324
tiropramide HCl		1983	19,324
argatroban	ANTITHROMBOTIC	1990	26,299
defibrotide		1986	22,319
cilostazol		1988	24,299
cloricromen		1991	27,325
enoxaparin		1987	23,333
ethyl icosapentate		1990	26,303
ozagrel sodium		1988	24,308
indobufen		1984	20,319
picotamide		1987	23,340
limaprost		1988	24,306
flutropium bromide	ANTITUSSIVE	1988	24,303
levodropropizine		1988	24,305
benexate HCl	ANTIULCER	1987	23,328
ecabet sodium		1993	29,336
enprostil		1985	21,327
famotidine		1985	21,327
irsogladine		1989	25,315
lansoprazole		1992	28,332
misoprostol		1985	21,329
nizatidine		1987	23,339
omeprazole		1988	24,308
ornoprostil		1987	23,339
pantoprazole sodium		1994	30,306
plaunotol		1987	23,340
polaprezinc		1994	30,307
rebamipide		1990	26,308
rosaprostol		1985	21,332
roxatidine acetate HCl		1986	22,326
roxithromycin		1987	23,342
sofalcone		1984	20,323
spizofurone		1987	23,343
teprenone		1984	20,323
tretinoin tocoferil		1993	29,348
troxipide		1986	22,327
didanosine	ANTIVIRAL	1991	27,326
famciclovir		1994	30,300
foscarnet sodium		1989	25,313
ganciclovir		1988	24,303
propagermanium		1994	30,308
rimantadine HCl		1987	23,342
sorivudine		1993	29,345
stavudine		1994	30,311
zalcitabine		1992	28,338
zidovudine		1987	23,345
alpidem	ANXIOLYTIC	1991	27,322
buspirone HCl		1985	21,324

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
etizolam		1984	20,318
flutazolam		1984	20,318
flutoprazepam		1986	22,320
metaclazepam		1987	23,338
mexazolam		1984	20,321
flumazenil	BENZODIAZEPINE ANTAG.	1987	23,335
bambuterol	BRONCHODILATOR	1990	26,299
doxofylline		1985	21,327
formoterol fumarate		1986	22,321
mabuterol HCl		1986	22,323
oxitropium bromide		1983	19,323
salmeterol hydroxynaphthoate		1990	26,308
APD	CALCIUM REGULATOR	1987	23,326
clodronate disodium		1986	22,319
disodium pamidronate		1989	25,312
gallium nitrate		1991	27,328
ipriflavone		1989	25,314
dexrazoxane	CARDIOPROTECTIVE	1992	28,330
bucladesine sodium	CARDIOSTIMULANT	1984	20,316
denopamine		1988	24,300
docarpamine		1994	30,298
dopexamine		1989	25,312
enoximone		1988	24,301
flosequinan		1992	28,331
ibopamine HCl		1984	20,319
milrinone		1989	25,316
vesnarinone		1990	26,310
amrinone	CARDIOTONIC	1983	19,314
xamoterol fumarate		1988	24,312
cefditoren pivoxil	CEPHALOSPORIN, ORAL	1994	30,297
brovincamine fumarate	CEREBRAL VASODILATOR	1986	22,317
nimodipine		1985	21,330
propentofylline		1988	24,310
succimer	CHELATOR	1991	27,333
trientine HCl		1986	22,327
fenbuprol	CHOLERETIC	1983	19,318
auranofin	CHRYSOTHERAPEUTIC	1983	19,314
aniracetam	COGNITION ENHANCER	1993	29,333
pramiracetam H_2SO_4		1993	29,343
nicorandil	CORONARY VASODILATOR	1984	20,322

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
dornase alfa	CYSTIC FIBROSIS	1994	30,298
neltenexine		1993	29,341
azosemide	DIURETIC	1986	22,316
muzolimine		1983	19,321
torasemide		1993	29,348
alglucerase	ENZYME	1991	27,321
cinitapride	GASTROPROKINETIC	1990	26,301
cisapride		1988	24,299
imiglucerase	GAUCHER'S DISEASE	1994	30,301
somatotropin	GROWTH HORMONE	1994	30,310
somatomedin-1	GROWTH HORMONE INSENSITIVITY	1994	30,310
pimobendan	HEART FAILURE	1994	30,307
erythropoietin	HEMATOPOETIC	1988	24,301
factor VIII	HEMOSTATIC	1992	28,330
malotilate	HEPATROPROTECTIVE	1985	21,329
buserelin acetate	HORMONE	1984	20,316
goserelin		1987	23,336
leuprolide acetate		1984	20,319
nafarelin acetate		1990	26,306
somatropin		1987	23,343
sapropterin hydrochloride	HYPERPHENYLALANINEMIA	1992	28,336
quinagolide	HYPERPROLACTINEMIA	1994	30,309
cadralazine	HYPERTENSIVE	1988	24,298
nitrendipine		1985	21,331
binfonazole	HYPNOTIC	1983	19,315
brotizolam		1983	19,315
butoctamide		1984	20,316
cinolazepam		1993	29,334
doxefazepam		1985	21,326
loprazolam mesylate		1983	19,321
quazepam		1985	21,332
rilmazafone		1989	25,317
zolpidem hemitartrate		1988	24,313
zopiclone		1986	22,327
acetohydroxamic acid	HYPOAMMONURIC	1983	19,313

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
sodium cellulose PO4	HYPOCALCIURIC	1983	19,323
divistyramine	HYPOCHOLESTEROLEMIC	1984	20,317
lovastatin		1987	23,337
melinamide		1984	20,320
simvastatin		1988	24,311
glucagon, rDNA	HYPOGLYCEMIA	1993	29,338
fluvastatin	HYPOLIPAEMIC	1994	30,300
acipimox	HYPOLIPIDEMIC	1985	21,323
beclobrate		1986	22,317
binifibrate		1986	22,317
ciprofibrate		1985	21,326
meglutol		1983	19,321
ronafibrate		1986	22,326
modafinil	IDIOPATHIC HYPERSOMNIA	1994	30,303
bucillamine	IMMUNOMODULATOR	1987	23,329
centoxin		1991	27,325
thymopentin		1985	21,333
filgrastim	IMMUNOSTIMULANT	1991	27,327
interferon gamma-1b		1991	27,329
lentinan		1986	22,322
pegademase bovine		1990	26,307
pidotimod		1993	29,343
romurtide		1991	27,332
sargramostim		1991	27,332
schizophyllan		1985	22,326
ubenimex		1987	23,345
cyclosporine	IMMUNOSUPPRESSANT	1983	19,317
gusperimus		1994	30,300
mizoribine		1984	20,321
muromonab-CD3		1986	22,323
tacrolimus		1993	29,347
sulbactam sodium	β-LACTAMASE INHIBITOR	1986	22,326
tazobactam sodium		1992	28,336
nartograstim	LEUKOPENIA	1994	30,304
pumactant	LUNG SURFACTANT	1994	30,308
telmesteine	MUCOLYTIC	1992	28,337
interferon β-1B	MULTIPLE SCLEROSIS	1993	29,339
afloqualone	MUSCLE RELAXANT	1983	19,313
doxacurium chloride		1991	27,326

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
eperisone HCl		1983	19,318
mivacurium chloride		1992	28,334
tizanidine		1984	20,324
naltrexone HCl	NARCOTIC ANTAGONIST	1984	20,322
tinazoline	NASAL DECONGESTANT	1988	24,312
clospipramine hydrochloride	NEUROLEPTIC	1991	27,325
nemonapride		1991	27,331
risperidone		1993	29,344
timiperone		1984	20,323
rocuronium bromide	NEUROMUSCULAR BLOCKER	1994	30,309
bifemelane HCl	NOOTROPIC	1987	23,329
choline alfoscerate		1990	26,300
exifone		1988	24,302
idebenone		1986	22,321
indeloxazine HCl		1988	24,304
levacecarnine HCl		1986	22,322
nizofenzone fumarate		1988	24,307
oxiracetam		1987	23,339
alendronate sodium	OSTEOPOROSIS	1993	29,332
beraprost sodium	PLATELET AGGREG. INHIBITOR	1992	28,326
epoprostenol sodium		1983	19,318
iloprost		1992	28,332
sarpogrelate HCl	PLATELET ANTIAGGREGANT	1993	29,344
trimetrexate glucuronate	*PNEUMOCYSTIS CARINII* PNEUMONIA	1994	30,312
histrelin	PRECOCIOUS PUBERTY	1993	29,338
gestodene	PROGESTOGEN	1987	23,335
nomegestrol acetate		1986	22,324
norgestimate		1986	22,324
promegestrone		1983	19,323
alpha-1 antitrypsin	PROTEASE INHIBITOR	1988	24,297
nafamostat mesylate		1986	22,323
adrafinil	PSYCHOSTIMULANT	1986	22,315
finasteride	5α-REDUCTASE INHIBITOR	1992	28,331
surfactant TA	RESPIRATORY SURFACTANT	1987	23,344

GENERIC NAME	INDICATION	YEAR INTRO.	ARMC VOL., PAGE
APSAC	THROMBOLYTIC	1987	23,326
alteplase		1987	23,326
tiopronin	UROLITHIASIS	1989	25,318
propiverine hydrochloride	UROLOGIC	1992	28,335
clobenoside	VASOPROTECTIVE	1988	24,300
cadexomer iodine	WOUND HEALING AGENT	1983	19,316
epidermal growth factor		1987	23,333