ADVANCES IN ANTIVIRAL DRUG DESIGN

Editor: E. DE CLERCQ

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Editor: E. DE CLERCQ
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PREFACE

Since the inception of the series on *Advances in Antiviral Drug Design* with Volume 1 in 1993, the antiviral chemotherapy field has continued to grow considerably. In addition to the established antiviral drugs (acyclovir, zidovudine, didanosine, zalcitabine, ribavirin, ganciclovir, and foscarnet), several new compounds have been approved (or may soon be approved): famciclovir and valaciclovir for the treatment of herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections; stavudine (D4T), saquinavir (Ro 318959), and lamivudine (3TC) for the treatment of retrovirus [i.e., human immunodeficiency virus (HIV)] infections; and cidofovir (HPMPC) for the treatment of human cytomegalovirus (HCMV) infections.

The purpose of the series on *Advances in Antiviral Drug Design* is to regularly review the "state of the art" on emerging new developments in the antiviral drug research field, thereby spanning the conceptual design and chemical synthesis of new antiviral compounds, their structure–activity relationship, mechanism and target(s) of action, pharmacological behavior, antiviral activity spectrum, and therapeutic potential for clinical use.

Volume 2 begins with a description of the antiviral potential of antisense oligonucleotides by J. Temsamani and S. Agrawal. According to the aims of the antisense technology, these oligonucleotides should be targeted at specific viral mRNA sequences so that translation to the virus-specified proteins is blocked; this has been achieved for a number of oligomers, some of which are now in clinical

trials for the treatment of HIV, HCMV, and human papilloma virus (HPV) infections.

Then C.-S. Yuan, S. Liu, S.F. Wnuk, M.J. Robins and R.T. Borchardt assess the role of S-adenosylhomocysteine (AdoHcy) hydrolase as target for the design of antiviral agents with broad-spectrum antiviral activity. This is followed by an in-depth account on the design and synthesis of a number of first-, second- and third-generation AdoHcy hydrolase inhibitors and their mode of action at the enzyme level.

V.E. Marquez provides a comprehensive description of the various carbocyclic (*carba*) nucleosides that have been synthesized and evaluated for antiviral activity. Although the number and diversity of the *carba*-nucleosides that have been found to be antivirally active (or inactive) is astonishingly high, there is no limit to further expansion of this fascinating class of molecules.

For the various nucleoside analogues that have to be intracellularly phosphorylated to the 5′-triphosphate stage, to interact with their target enzyme (i.e., herpesviral DNA polymerase or retroviral reverse transcriptase) the first phosphorylation step is often the rate-limiting step, and thus various strategies are envisaged by C. Périgaud, J.-L. Girardet, G. Gosselin and J.-L. Imbach on how to bypass this initial phosphorylation and to deliver the nucleoside 5′-monophosphate directly inside the cells.

The HIV protease has been considered as a paradigm for rational drug design. The enzyme is among the best understood in terms of both structure and action, and, because of its crucial role in the maturation of HIV, it has been vigorously pursued as a target for anti-HIV chemotherapy. In their comprehensive review of the multidisciplinary approach towards the development of HIV protease inhibitors A.G. Tomasselli, S. Thaisrivongs and R.L. Heinrikson highlight those protease inhibitors which have been brought forward to clinical trials.

The contributors to Volume 2 of *Advances in Antiviral Drug Design* have attempted to follow the path initiated by their predecessors in Volume 1—that is to provide a comprehensive and thorough account on what has been recently achieved, or conceived, in selected areas of antiviral research enjoying enormous current momentum. I hope this volume, like the first, will be of great value to all of those that endeavor at, or are interested in, the fundamental approaches towards the. chemotherapy of viral diseases.

E. De Clercq
Editor

ANTISENSE OLIGONUCLEOTIDES AS ANTIVIRAL AGENTS

Jamal Temsamani and Sudhir Agrawal

I. INTRODUCTION

Antisense oligonucleotide technology has generated considerable enthusiasm in the research and medical community because of its specificity and the breadth of its potential applications. The recent commencement of clinical trials using antisense oligonucleotide therapy for viral illnesses, including papilloma virus, human immunodeficiency virus (HIV), cytomegalovirus, and cancer, has heralded a new era in drug design.[1-6]

Antisense oligonucleotides are small synthetic pieces of single-stranded DNA. Their ability to selectively inhibit gene expression led to the suggestion that they would be useful therapeutic agents. Gene expression is inhibited by hybridization of the oligonucleotide, to sequences in the gene or the messenger RNA (mRNA) target by Watson–Crick base pairing in which adenosine and thymidine or guanosine and cytidine interact through hydrogen bonding. These simple base-pairing rules govern the interaction between the antisense oligonucleotide and the target nucleic acid, allowing the design of oligonucleotides to target any gene of a known sequence. A major advantage of this strategy over the use of conventional drugs is in the potential specificity of action of antisense oligonucleotides. In principle, an oligonucleotide of a specified sequence of 17 nucleotides or more can be designed to target specifically any single gene within the human genome. Additionally, achieving inhibition at the gene or mRNA level is believed to be a much more efficient intervention in the disease process than inhibition at the protein level. The use of antisense oligonucleotides as antiviral agents seems simple. The genome of viruses is much smaller than that of humans, making a choice of target relatively straightforward; and the viral genome and the mammalian genome code for different functions, so that targeting the viral genome will not interfere with the expression of host sequences.

The first example of specific inhibition of gene expression by an oligonucleotide was reported by Zamecnik and Stephenson,[7,8] who demonstrated that a short

oligonucleotide inhibited Rous sarcoma virus replication in cell culture. Since then, the field has progressed enormously. The development has been driven largely by the ability to synthesize oligonucleotides and their modified analogs, in reasonable quantity and of good purity, through automated synthesis.[9,10] Numerous studies have demonstrated the ability of antisense oligonucleotides to modulate gene expression. In this chapter, we have selected studies in several therapeutic areas to demonstrate the antiviral effect of oligonucleotides.

II. OLIGONUCLEOTIDE APPROACHES

The oligonucleotide approaches include the following (Figure 1):

Antisense oligonucleotides. Oligonucleotides (short segments of DNA or RNA) bind to target single-stranded RNA at a specific site by Watson–Crick base-pairing and, through a variety of mechanisms, prevent protein production.
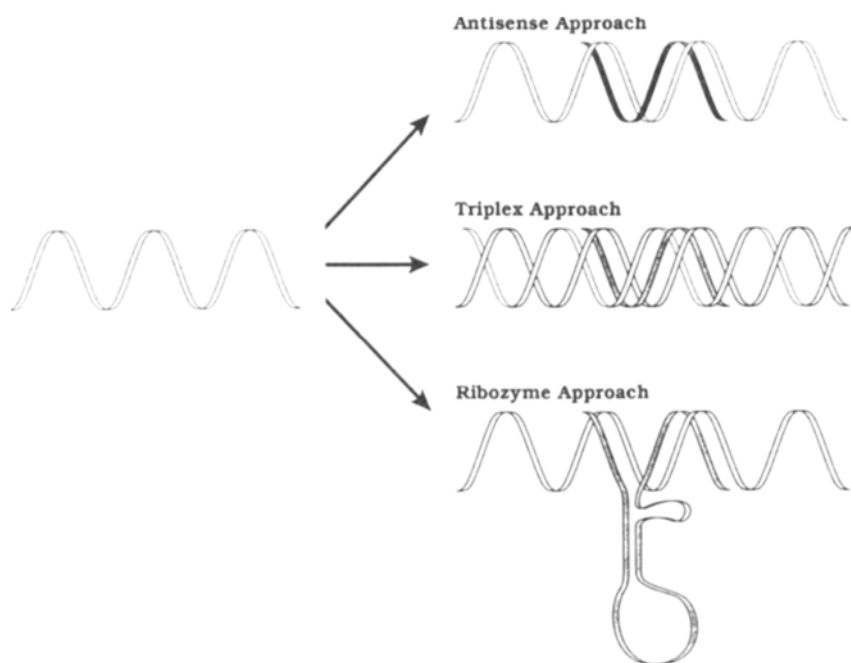

Figure 1. Oligonucleotide approaches. In the antisense approach, the oligonucleotide binds to target RNA by Watson-Crick base-pairing. In the triplex approach, the oligonucleotide binds to ds-DNA by Hoogsteen hydrogen bonding, forming triple-stranded structure. In the ribozyme approach, the oligonucleotide is an RNA molecule that has RNA-cleaving capability.

Triplex approach. The oligonucleotide binds to double-stranded helical DNA by Hoogsteen hydrogen bonding.[11] The formation of the triple-stranded structure (triplex) prevents transcription to mRNA, thereby preventing protein production. Targeting the origin of disease, the gene, benefits from having severalfold fewer sites to block but requires the drug not only to penetrate into nucleus of the cell, but also to penetrate the chromatin that surrounds DNA.

Ribozyme approach. Ribozymes are RNA molecules that have RNA-cleaving capability.[12] Ribozymes can be delivered either exogenously or by using gene therapy approaches. There is a continued need for improving ribozyme stability and production methodology.

This chapter focuses on the use of antisense oligonucleotides targeted at mRNA. Although the triplex and ribozyme approaches appear to have great promise, further investigations are needed before they can be employed routinely.

III. OLIGONUCLEOTIDE ANALOGS

Although the naturally occurring phosphodiester oligonucleotides have displayed activities against some targets in vitro,[13-15] the key to the future value of oligonucleotide therapeutics resides in the development of modified oligonucleotides. Phosphodiester oligonucleotides, despite their solubility and high affinity, are rapidly degraded in cell culture media and in vivo, with a half-life in animals of about 5 minutes, which limits their use as therapeutic agents.[13,16-18] Several modified versions of the naturally occurring phosphodiester oligonucleotides have been synthesized (Figure 2) and tested in cell culture and in vivo. The goal is to increase the half-life of the oligonucleotide while maintaining its ability to cross the cell membrane and to bind with high affinity and selectivity to the target RNA. Although any one of the three components of an oligonucleotide (the base, sugar, and phosphodiester backbone) could be modified in principle, the major modifications have been carried out on the phosphodiester backbone.

A. Phosphorothioate

Phosphorothioate oligonucleotides are among the most obvious and thus probably the earliest used analogs of naturally occurring phosphates and are still the most widely applied analogs of oligonucleotides for antisense applications.[5,19,20] In a phosphorothioate oligonucleotide one of the phosphate oxygen atoms not involved in the bridge is replaced by a sulfur atom, with the negative charge being distributed asymmetrically and located mainly on sulfur.[20-22] Thus the molecule has the same charge as the parent compound. Replacement of a nonbridging oxygen atom by a sulfur produces a chiral center at the phosphorus, which leads to the formation of diastereomers. This substitution markedly decreases the rate of oligonucleotide degradation, extending the half-life in vitro and in vivo.[23-26]
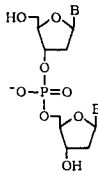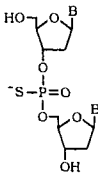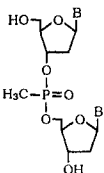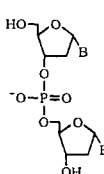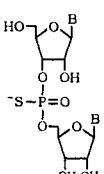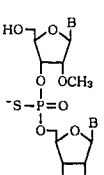
Structure	Duplex Stability	*In vitro* and *In vivo* Stability	RNaseH Activity	Ref.
Phosphodiester	+++	−	yes	(13)
Phosphorothioate	++	+++	yes	(20)
Methylphosphonate	++	+++	no	(33)
α-oligonucleotide	+++	+++	no	(37)
Phosphorothioate oligoribonucleotide	++++	++	no	(167)
2'OMe phosphorothioate oligoribonucleotide	++++	+++	no	(168)

Figure 2. Structure of different modified oligonucleotides.

Oligonucleotide Design	Duplex Stability	In vitro and In vivo Stability	RNaseH	Antiviral Activity
5' 3'				
X—▬▬▬—X Capped	+++	+++	yes	(60, 62,160)
Chimeric	+++	++++	yes	(82)
Hybrid	++++	++++	yes	(99)
Self-stabilized	+++	++++	yes	(41, 130)

Figure 3. Design of oligonucleotides.

Resistance of phosphorothioates to degradation by nucleases could be due to the lack of binding of the oligonucleotides to these enzymes. The phosphorothioate oligonucleotides are relatively easy to synthesize, requiring sulfurization only instead of the normal oxidation step as the last step in the cyclic synthesis. They hybridize with the target nucleic acid with lower melting temperatures than the naturally occurring oligonucleotides and are capable of activating RNase H, a characteristic essential for good antiviral activity.[20,23,27] The phosphorothioate oligonucleotides appear to enter cells readily[28–30] and are found in most organs when injected in animals.[17,26,30,31,32]

B. Methylphosphonate

The limited ability of charged oligonucleotides to cross the cell membrane has led to the development of a class of nonionic chemical analogs, called methylphosphonates, in which the negatively charged phosphate oxygen is replaced by a neutral methyl group.[33] Methylphosphonate oligonucleotides are highly lipophilic, resistant to nucleases, and readily taken up by cells.[34,35] However, they have poorer hybridization characteristics and are less soluble than charged oligonucleotides. The major drawback of these analogs is that they do not activate RNase H, thus lacking an important catalytic effect on target RNA. Although the antiviral activity of these oligonucleotides is well documented,[33] the concentrations required to induce a biological effect are often high. The high concentrations required to inhibit gene expression and the poor solubility of methylphosphonate oligonucleotides will likely limit their usefulness.[36]

C. Other Modifications

Additional modifications to the base, sugar, and phosphate moieties could result in enhanced therapeutic properties of oligonucleotides.[13,37] The α-anomeric oligonucleotides result from the rotation of the heterocyclic base and its bond to the sugar moiety[38] (Figure 2). They are resistant to nuclease degradation but hybridize

to their target nucleic acid with less avidity than naturally occurring phosphodiester oligonucleotides.[39] Additionally, these compounds do not activate RNase H.

Another interesting strategy is to prepare mixed-backbone oligonucleotides, capitalizing on the ideal aspects of each type of nucleotide analog. For example, we and others[27,40] have synthesized oligonucleotides containing methylphosphonate backbones at the 5' and the 3' ends of the oligonucleotide, with a central core of either phosphorothioate or phosphodiester composition (chimeric oligonucleotides; Figure 3). The methylphosphonate linkages increase the uptake and stability of the oligonucleotide, whereas the central core enhances the hybridization and activation by RNase H. The 3' and 5' ends of the oligonucleotide can also be replaced by ribonucleotides, which results in enhanced affinity and specificity (hybrid oligonucleotides; Figure 3).

Another design of oligonucleotides is to self-stabilize them against nucleases, which is accomplished by structural modification rather than chemical modification.[41] The self-stabilized oligonucleotide has two domains: a single-stranded antisense sequence and a hairpin loop at the 3' end (Figure 3), which reduces the accessibility of the oligonucleotide to nucleases. In the presence of the target nucleic acid, the hairpin loop is destabilized and the whole antisense sequence binds to the target. The oligonucleotides can also be stabilized by coupling them with a chemical moiety (capped oligonucleotides; Figure 3).[42] This chemical group can be coupled either at the 5' or at the 3' end of the oligonucleotide, but conjugating at the 3' end produces a more stable compound. A judicious choice of the chemical group (e.g., hydrophobic group) can also increase cellular uptake of the oligonucleotide.

IV. SYNTHESIS OF OLIGONUCLEOTIDES AND ANALOGS

Advances in oligonucleotide synthetic chemistry have contributed significantly to the growing use of antisense oligonucleotides. Solid-phase methods using phosphotriester, phosphoramidite, or H-phosphonate chemistry are the most common methods for synthesis of oligonucleotides and their various analogs.[9,10] There are three essential steps in solid-phase synthesis: (i) attachment of the first nucleoside to a solid support, (ii) assembly of the oligonucleotide chain, and (iii) deprotection and removal of the oligonucleotide from the solid support (Figure 4).

Nucleosides used in solid-phase synthesis are protected; base-labile acyl groups are used to protect the exocyclic amino groups of adenine, cytosine, and guanine, and an acid-labile dimethoxytrityl group is used to protect the 5'-hydroxyl group. Oligonucleotides are synthesized from the 3' end to the 5' end. Each nucleoside addition involves removal of the acid-labile dimethoxytrityl group coupling of the activated monomer and appropriate washing and oxidation steps. This cycle is repeated to extend the oligonucleotide chain to the desired length (Figure 4). Deprotection is then carried out under basic conditions to cleave the oligonucleotide from the solid support and to remove base-protecting groups.
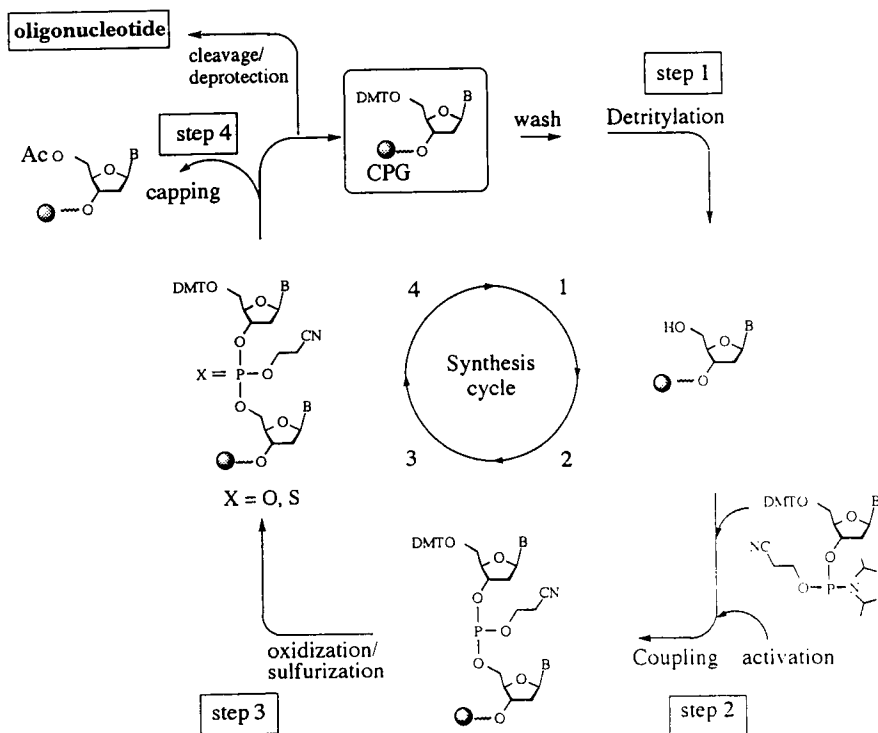
Figure 4. Stepwise synthesis of oligonucleotides by phosphoramidite approach.

The phosphotriester method, which dominated oligodeoxynucleotide synthesis for a long time, is no longer the method of choice because of side reactions and low coupling efficiencies. With this approach, oligonucleotides containing phosphate[43] and phosphorothioate[44] internucleotide linkages have been synthesized. Phosphoramidite[45] and H-phosphonate[46,47] approaches are currently the preferred methods for assembling oligonucleotides and their sugar-phosphate backbone modified analogs.[48] Ionic oligonucleotides can be purified by using high-performance liquid chromatography (HPLC) or polyacrylamide gel electrophoresis (PAGE). For methylphosphonate oligonucleotides, which are not charged, reverse-phase HPLC can be used for purification.

V. ANTISENSE OLIGONUCLEOTIDES: WHAT MUST BE CONSIDERED?

To induce the biological effect, the oligonucleotide must meet the following requirements: (1) it must have a sufficiently long half-life in the biological medium

and inside the cells to be able to display its desired action inside the cell; (2) it must cross the cell membrane to reach its target sequence; and (3) it must bind to its target nucleic acid specifically and with high affinity.

A. Stability

A major requirement to be met by antisense oligonucleotides is that they remain stable both in vitro and in vivo. The stability of the oligonucleotide in tissue culture or in vivo will influence its final biological effect. Phosphodiester oligonucleotides are susceptible to degradation by nucleases found in biological media and inside cells.[49,50] In vivo, these oligonucleotides have a half-life of about 5 minutes, which limits their use as antiviral agents.[17,18,51] Since the internucleotide linkage is the site of attack by enzymes, modifications made in the internucleotide linkage can improve stability of the oligonucleotide. Modification of the phosphodiester linkage into phosphorothioate or methylphosphonate linkages enhances resistance to nucleases.[20,33,52] Phosphorothioate oligonucleotides are resistant to purified exo- and endonucleases, and they exhibit nuclease resistance in intact cells and in animal tissues. Oligonucleotides containing α-anomers in the sugar moiety are also resistant to nuclease attack.[39] Modification at the 2' position of the sugar also enhances the stability of the oligonucleotide. In addition to uniform modifications, a number of groups conjugated at the 3' end of the oligonucleotide have been reported to enhance nuclease resistance.[42,50,53,54] The predominant nuclease activity present in cell culture systems and in animal tissues is a 3'-exonuclease, since the 3'-capped oligonucleotides are more resistant.[42]

B. Uptake

Despite its critical importance, oligonucleotide uptake remains poorly understood. Oligonucleotides are polyanionic (with the exception of methylphosphonate oligonucleotides) and have a molecular weight in the range of 6000 to 9000 (for 17 to 28 mers). They cannot passively diffuse across the cell membrane, but many studies have shown biological effects with antisense oligonucleotides, demonstrating that they do indeed enter cells. The first investigations into the mechanism of cellular uptake showed that a cell surface protein of about 80 kDa is involved in the uptake of oligonucleotides, but the true role of this protein is still unknown.[55] From the studies published to date using either fluorescent or radiolabeled oligonucleotides, it is clear that cellular uptake is sequence-dependent, is a saturable process, and is dependent on temperature and energy.[28-30,56,57] The uptake is also cell-dependent.[30] We studied the cell uptake of a fluorescent labeled 25-mer phosphorothioate oligonucleotide (GEM91) in different cell lines (Figure 5). Not only the uptake but the intracellular distribution was cell-dependent as well. In some cells, the oligonucleotide was localized in cytoplasmic vesicles, whereas in others it was diffused. It is believed that phosphodiester and phosphorothioate oligonucleotides enter the cell by adsorptive endocytosis and/or fluid phase endocytosis,

A.
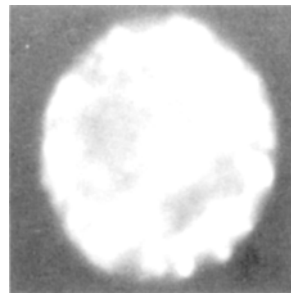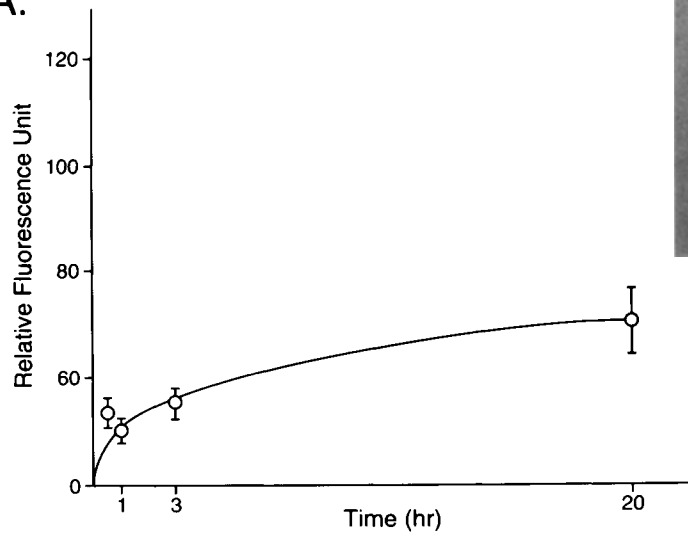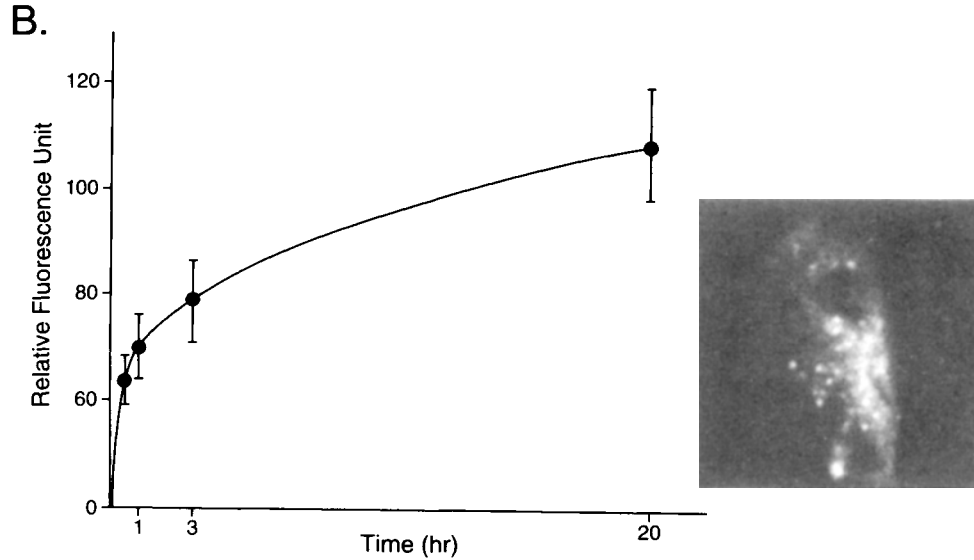
Figure 5. Cell uptake. A fluorescently labeled 25-mer phosphorothioate oligonucleotide (GEM91) was incubated with MOLT-3 cells (**A**), monocyte-derived macrophages (**B**), and primary human lymphocytes isolated from a normal donor (**C**). At each time point, the uptake was measured by flow cytometry. At 20 hours, the intracellular distribution was analyzed by laser-assisted confocal microscopy. (Reprinted from Ref. 90.) (*continued*)

C.
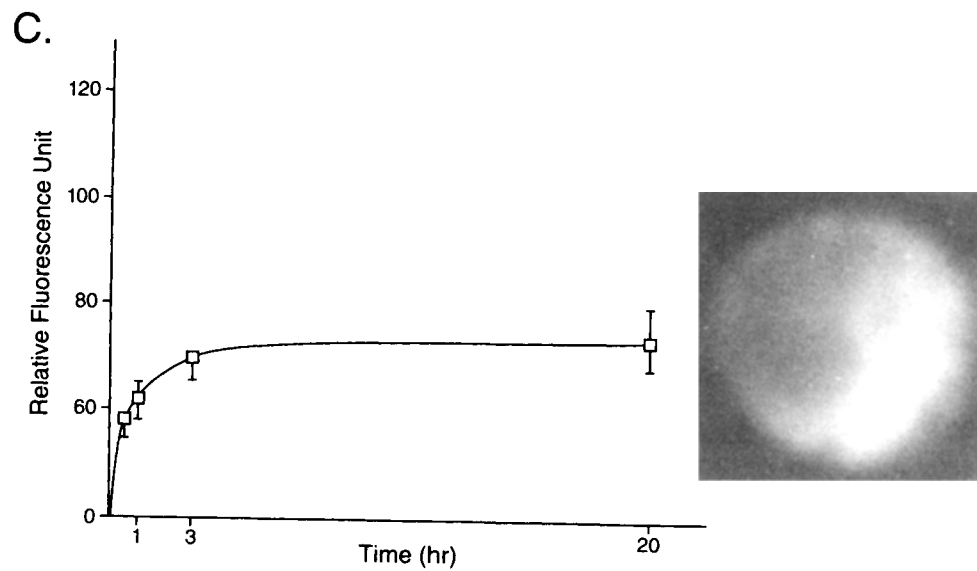
Figure 5. (Continued)

whereas cellular uptake of methylphosphonate oligonucleotides may involve either passive diffusion or adsorptive endocytosis.[28,56,58,59] Once the oligonucleotide crosses the cell membrane, it is distributed in the cytoplasm and in the nucleus. In most of the studies published to date, the concentration in the cytoplasm is significantly greater than in the nucleus. There has been much interest in improving oligonucleotide uptake. This includes conjugation of oligonucleotides with hydrophobic or lipophilic molecules[60-62] and cationic molecules.[63,64]

C. Affinity

The complex formed between the oligonucleotide and its target nucleic acid must be sufficiently stable under physiological conditions to block the translation of the target mRNA. The affinity of an oligonucleotide to its target sequence results from the hybridization interaction, which for antisense oligonucleotides is Watson–Crick base-pairing. The complex formed between the oligonucleotide and its target is characterized by the melting temperature (T_m) of the duplex. As with any other drug–receptor interactions, pharmacological activity requires a minimum level of affinity. The affinity of an oligonucleotide is determined by the length of the oligonucleotide, increasing as the oligonucleotide gets longer. Affinity also varies as a function of the sequence of the duplex, increasing with the number of G-C base pairs. For many targets, the minimum length of an oligonucleotide is approximately 15–17 nucleotides.

D. Specificity

One of the basic requirements to be met by antisense oligonucleotides is absolute specific binding to the target sequence. The specificity derives from the selectivity of the base-pairing. Statistically, the sequence of a 17-mer oligonucleotide occurs just once in the human genome. In the case of viral infections, a priori, the specificity should not be an issue because viral nucleic acids code for different functions than do their mammalian counterparts. Targeting the viral genome should therefore not interfere with the expression of host sequences. Specificity may also be gained by judicious selection of target sites to take advantage of target structure and the activity of RNase H. Oligonucleotides also display nonspecific effects, however, especially at higher concentrations.

VI. MECHANISM OF ACTION

Oligonucleotides are designed to modulate the genetic expression from the gene to protein. Figure 6 summarizes these processes. The process of genetic expression begins with the transcription of DNA to RNA. The RNA polymerase recognizes specific start sequences on DNA (promoter regions), and one strand of the DNA (the antisense strand) is transcribed into a sense pre-mRNA molecule. The 5′ end of this pre-mRNA is then capped by 7-methylguanosine. Most pre-mRNA species
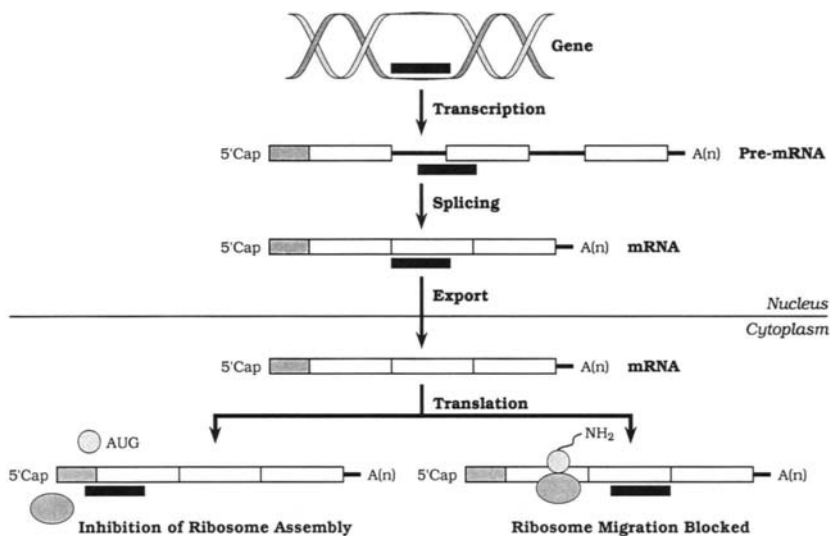
Figure 6. Mechanism of action of oligonucleotides. Oligonucleotides can interrupt the process of genetic expression by different mechanisms. The oligonucleotide can inhibit transcription of the gene, splicing of pre-mRNA, or translation of mRNA.

are also polyadenylated at the 3' end by a post-transcriptional mechanism. The poly(A) sequence plays an important role in the stabilization, transport, and maturation of the RNA. Eukaryotic genes usually contain intervening sequences called "introns," which, in most pre-mRNA species, are excised during splicing. The mature RNA is then exported to the cytoplasm, where it is translated into proteins.

The potential sites of action and mechanisms by which oligonucleotides can interrupt the process of gene expression are numerous (Figure 6). The precise mechanism of action inside intact cells is, however, still largely a matter of conjecture.

A. Inhibition of Transcription

Oligonucleotides may bind to DNA and prevent either initiation or elongation of transcription by preventing effective binding of factors required for transcription. Oligonucleotides can also bind to segments of DNA that are partially unwound by the transcription complex. Recently we have shown in vitro that oligonucleotides complementary to the antisense strand of DNA can inhibit transcription, whereas oligonucleotides complementary to the sense strand failed to do so.[65] Also, Hélène and colleagues[66] reported that short oligonucleotides with acridine derivatives at the 3' terminus inhibit the transcription of the β-lactamase gene. An alternative to

binding to single-stranded DNA is to inhibit transcription by interacting with double-stranded DNA through formation of triple-stranded structures.[11,67] To form triple-stranded structures, the oligonucleotide must bind to dsDNA by hydrogen bonds other than Watson–Crick bonds. The oligonucleotide becomes a third strand in the complex by recognizing hydrogen bonding sites on a purine strand.

The process of inhibition of transcription is of great interest to researchers, mainly because inhibition at this level should be more efficient than at the level of translation, since many copies of mRNA are produced from one copy of DNA. This strategy is hampered, however, by the fact that oligonucleotides must cross the nucleus and have access to DNA within the chromatin.

B. Inhibition of Splicing

A key step in the maturation of the RNA is the excision of introns during the splicing reaction. Oligonucleotides designed to bind to the pre-mRNA across the boundary between the exons and introns (splice sites) may inhibit the splicing reaction by preventing the assembly of the spliceosome or by impeding the ability of the spliceosome to cleave the RNA.[68,69] Alternatively, the formation of a double-stranded oligonucleotide-RNA complex may activate RNase H. Targeting the exon-intron boundaries would limit the activity of the oligonucleotide to binding only to pre-mRNA transcripts. Evidence of this type of gene regulation has been demonstrated by inhibition of viral replication with oligonucleotides complementary to splice sites.[70–73]

C. Inhibition of Translation

Most antisense oligonucleotides to date have been designed with the aim of inhibiting translation of mRNA to proteins. Oligonucleotides can be designed to bind to the translation initiation codon. In this way, the RNA-oligonucleotide duplex may prevent binding of the ribosomes and the initiation of translation by a direct steric effect. Oligonucleotides can also be targeted to coding sequences downstream of the initiation codon, thereby inhibiting the elongation and translocation of the ribosomes along the mRNA. Oligonucleotides targeted to these regions seem to be less efficient in inhibiting translation than those targeted to the initiation site, suggesting that the ribosomes are able to destabilize the double-stranded structures formed between the oligonucleotide and the mRNA. Most of the studies to date have used oligonucleotides targeted to the regions overlapping the initiation codon. RNase H, a ubiquitous enzyme that recognizes the double-stranded mRNA-oligonucleotide structure and cleaves the mRNA, rendering it nonfunctional, is important in inhibiting translation (see below).

D. Other Effects

Oligonucleotides may also inhibit the process of genetic expression by other mechanisms such as disrupting the structure or stability of the RNA. Most of the RNAs adopt a variety of three-dimensional structures, such as the stem-loop, which play a crucial role in the functions of RNAs. Binding of oligonucleotides to the RNA can disrupt the stem-loop structure and alter the function of the RNA.[74] Oligonucleotides can also be targeted to the 5′ and 3′ untranslated regions,[7,8] which are important for the stability of the RNA.

E. Activation of RNase H

RNase H is an endonuclease that recognizes the heteroduplex RNA/DNA and degrades the RNA moiety.[75] It is believed that some antisense oligonucleotides function, at least in part, through activation of RNase H, resulting in degradation of the target RNA. Recognition of the crucial importance of RNase H for the activity of oligonucleotides came from one major observation. Methylphosphonate, α-anomeric oligonucleotides, oligoribonucleotides, and analogs, which do not activate RNase H, were unable to inhibit translation of proteins, despite their high binding affinity and stability,[33,76,77] whereas the phosphodiester and phosphorothioate analogs were much more efficient at inhibiting translation. A wealth of literature supports the correlation between the efficacy of oligonucleotides in inhibiting mRNA translation and the induction of mRNA cleavage by RNase H. The cleavage of RNA by RNase H plays a crucial role in antisense activity because it allows the oligonucleotide to move to another target, effectively recycling the oligonucleotide and allowing lower concentrations of oligonucleotide to achieve biological activity.

VII. ANTIVIRAL TARGETS

Since the first experiments of Zamecnik and Stephenson,[7,8] oligonucleotides have been designed for use against many different viruses (Table 1 and 2), including many important human viruses. Viruses are attractive therapeutic targets since their genome is much smaller than that of humans and their genetic sequences are unique with respect to the human host; therefore, oligonucleotides that target the viral genome will not interfere with the expression of host sequences. Human immunodeficiency virus (HIV) has been a major target because of its considerable medical, social, and economic consequences.

A. Human Immunodeficiency Virus

HIV is the etiologic agent of the acquired immunodeficiency syndrome (AIDS).[78–80] The urgent need for chemotherapy of AIDS has made HIV the major target for antisense oligonucleotides. The genome organization of HIV is more

Table 1. In Vitro Anti-HIV Activities of Antisense Oligonucleotides

Virus	Target	Cells	Oligo	Length	Effective concentration (μM)	Ref
HTLVIII-B	5' end, others	H9	PO	12–26	2–10	7, 8
HTLVIII-B	20 sites	H9, MOLT-3	PO	20	3–15	71
HTLVIII-B	5' end, tat	H9, MOLT-3	PS, others	15–20	3	25
HTLVIII-B	tat	H9, MOLT-3	PO-Pm	15–20	0.5–13	82
HTLVIII-B	tat	H9	Pm	8	100	83
HTLVIII-B	rev, Sd(C)28	ATH8	PS	14–28	<1	84
HTLVIII-B	rev, tat	MT-4	PO-PS	20	0.02–0.5	86
HTLVIII-B	tat	CEM	PO-PS	28	5–25*	85
HTLVIII-B	TAR	CEM	PO, PS	18–28	<1, >10*	118
HIV-1	LTR, tat	MT4	PO-PLL	18	0.2	112
HTLVIII-B	tat	MOLT-3	PS-Chol	7–20	<1	60
HTLVIII-B	—	CEM, MOLT-3	PS-Chol	10	0.8	109
HIV-I_{RF}	gag, pol, env	H9	PS	22–27	0.1–2, 30*	87
HTLVIII-B	—	MOLT-3	P(S)2	8–28	0.5–3	97
HTLV-1	tat	MT4	PS, others	12	0.25–60	98
HTLVIII-B	tat	MOLT-3	PS-2'OMe	20	0.1	99
HIV-1	—	Hut-78	PS	8	<1	93
HTLVIII-B	tat, RRE, rev, gag	MOLT-3	PS	28	1**	88, 89
HTLVIII-B	RRE	MOLT-3	PS	15–28	0.1–0.5	91
HTLVIII-B	gag	PBMCs	PS	25	0.25–1	90
HIV-1/Bal	vpr	Macrophages	PS	27	10–20	92
HIV-1/Bal	gag	Macrophages	PS	25	1	90

Notes: PO, phosphodiester; PS, phosphorothioate; Pm, methylphosphonate; Chol, cholesterol; PLL, poly(L-lysine); P(S)2, phosphorodithioate; PS-2'OMe, phosphorothioate with segments of 2'-O-methylribonucleotide linkages; PBMCs, peripheral blood mononuclear cells.
*Chronically infected cells were used.
**Long-term experiment.

complex than that of other retroviruses (Figure 7, top). In addition to gag, pol, and env proteins, HIV produces several others. Most of these proteins are not associated with the virion but are involved in intracellular steps of the viral life cycle. Two of these proteins, tat and rev, are essential for viral replication.

Numerous oligonucleotides have been tested for their ability to inhibit HIV replication in cell culture systems, including phosphodiester,[71,81] methylphosphonate,[82,83] phosphorothioate,[25,84–93] and other classes of oligonucleotides.[25,94–99] These oligonucleotides are targeted to various regions of HIV genome, including the 5' cap site, the 5' noncoding region, AUG sites, splice sites, tRNA binding sites, polyadenylation signal, etc. Although inhibition of processing/translation of HIV-1 by antisense oligonucleotides is the principal activity, oligonucleotides (especially phosphorothioates) have been shown to inhibit HIV by other mechanisms.

Table 2. *In Vitro* Antiviral Activities of Antisense Oligonucleotides

Virus	Target	Cells	Oligo	Length	Effective concentration	Ref
Herpes simplex	Vmw65	LTK⁻	PO	18	150 nM	123
	IE 22,47	Vero	α-PO-Psor	13	0.5 μM	124
	IE 4,5	Vero	Pm	9, 12	15–100 μM	70, 72
			Pm-Psor		5 μM	
	UL13	Hela	PS	21	4 μM	52
	—	Hela	PS	28	1 μM	128
	ICP4	Vero	PS(SS)	32	5 μM	130
Influenza	3′ end, PB1	MDCK	PO	20	>80 μM (PO)	131, 132
			PS	20	1.25–15 μM (PS)	
	PB1	MDCK	PS (SS)	33	5 μM	130
Human papilloma	E6/E7	CaSki	PS	18–33	1–5 μM	141
	E6/E7	Oral cancer	PO	20	1–5 μM	142
	E2	C127	PS	20	5–7 μM	3
	E2	C127	PS	20	10–100 nM	3
Epstein-Barr	DP, EBNA-1	H1	PS	28	1 μM	147
	EBNA-1	Lymphoblastoid	PO	15	10 μM	148
Hepatitis B	polyA signal	HepG2	PS	21	50 μM	153
	HBₛAg	Hepatocellular carcinoma	PO	12–15	17 μM (PO)	152
			PS		5 μM (PS)	
Hepatitis B (duck)	pre-S	Hepatocytes	PS	15–17	1.5 μM	155
Hepatitis C	5′ region	Cell free system	PO	27–28	1 μM	154
Cytomegalovirus	IE1/IE2	NHDF	PS	20–21	0.37 μM	2
	UL36/37	Human foreskin fibroblasts	PS	20	0.08 μM	156
Vesicular s tomatitis	N, NS, G	Mouse L	Pm	9	50–100 μM	159
	N	L929	PO-PLL	15	>1 μM	160, 161, 163
	N, NS, G	L929	PO-lipid	15	50–150 μM	62
Rous sarcoma	3′ region	Chicken fibroblasts	PO	13	2 μM	7, 8

Note: PO, phosphodiester; PS, phosphorothioate; Pm, methylphosphonate; Psor, Psoralen; PLL, poly(L-lysine); SS, self-stabilized.

HIV-1

Oligonucleotide Phosphorothioates

gag-28	C G C T C T C G C A C C C A T C T C T C T C C T T C T A	776-802
gag-24	C T C T C G C A C C C A T C T C T C T C C T T C	778-800
rev1-28	T C G T C G C T G T C T C C G C T T C T T C C T G C C A	5970-5997
rev2-28	C T G T C T C C G C T T C T T C C T G C C A T A G G A G	5976-6003

Figure 7. Long-term inhibition of HIV. Top: HIV genomic organization. Bottom: (**A**) Cells infected with HIV were treated twice a week with 1 µM oligonucleotide. Every 3 days, the level of p24 was measured. (**B**) At day 35, an aliquot of cells treated with 1 µM oligonucleotide was split and maintained without antisense oligomer. (**C**) An aliquot of cells treated with 1 µM oligomer was split and incubated with 0.01 µM oligonucleotide. (**D**) An aliquot of cells treated with 0.1 µM was split at day 53 and incubated with 0.01 µM oligonucleotide. (Reprinted from Ref. 89.)

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In early studies, the naturally occurring phosphodiester oligonucleotides were used. The effect of oligonucleotides complementary to the tRNALys primer binding site and the splice sites of the *tat* region was assessed.[81] The oligonucleotides were added to H9 cells (transformed T cells) at the time of infection by HIV-1 (HTLV-IIIB). Four days later, the viral replication was monitored by measuring reverse transcriptase activity and the expression of viral proteins p17 and p24. The oligonucleotide complementary to the splice acceptor site of tat was found to be the most potent, with an IC_{50} in the range of 2–10 μM. In consecutive studies, 20-mer phosphodiester oligonucleotides complementary to 20 different regions of HIV were studied.[71] In comparing the activity of the different oligonucleotides, it was found that oligonucleotides complementary to the 5′ end region and to the polyadenylation signal were the most potent.

As described earlier, phosphodiester oligonucleotides are unstable in culture media and in cells, which limits their use as antiviral drugs. To stabilize the oligonucleotides, nuclease resistant methylphosphonate linkages were incorporated in the oligonucleotide complementary to the *tat* region.[82] Not only did the stability of the oligonucleotide increase but the efficacy increased as well: the methylphosphonate oligonucleotide was four- to fivefold more potent than the phosphodiester analog. The introduction of methylphosphonate linkages also reduced the cytotoxicity of the oligonucleotide. Zaia et al.[83] also targeted the splice acceptor site of *tat* using an 8-mer methylphosphonate oligonucleotide. H9 cells were incubated with the oligonucleotide 1 hour prior to infection with HIV-1. At 100 μM, syncytium formation and reverse transcriptase activity were inhibited in a specific manner. The sense oligonucleotide exerted a slight inhibition and the control oligonucleotide was not effective. Kim et al.[86] and Daum et al.[73] compared the activity of unmodified oligonucleotides and phosphorothioate-capped oligonucleotides (at both ends). Both groups found that phosphorothioate-modified oligonucleotides were more active than the unmodified oligonucleotides.

Phosphorothioate oligonucleotides are probably the most widely used analogs for HIV inhibition.[5,100–103] Different phosphorothioate oligonucleotides complementary to the 5′ end region of HIV and to the splice sites of *tat* were incubated with H9 or MOLT-3 cells in the presence of HIV-1.[25] All of the oligonucleotides, including the noncomplementary oligonucleotides, inhibited HIV replication at 3 μM. Similar results were obtained by Matsukura et al.,[84] who found that phosphorothioate analogs complementary to HIV sequences, as well as noncomplementary analogs including homo-oligomers, exhibited potent antiviral activity. A 28-mer phosphorothioate oligonucleotide deoxycytidine [Sd(C)28] inhibited de novo viral DNA synthesis at 1 μM; however, this compound failed to prevent the translation of gag proteins. The authors argued that this compound could block viral replication prior to or at the stage of proviral DNA. Interestingly, it was found that Sd(C)28 is a powerful inhibitor of HIV reverse transcriptase.[104,105] Other homo-oligomers were also found to inhibit HIV replication.[106–108]

Phosphorothioate oligonucleotides are also targeted against the rev-responsive element (RRE), a region required for the activity of rev protein.[91] MOLT-3 cells were infected with HIV-1 (HTLV-IIIB), and the oligonucleotides were added 2 hours later. The activity of 28-mer anti-RRE phosphorothioate oligonucleotide was compared to the activity of a control oligomer. Four days after the infection, it was found that there was a specific and dose-dependent inhibition of syncytium formation at low doses (0.1 and 0.5 μM), but when the dose was increased to 10 μM there was nonspecific inhibition. Morvan et al. compared the antiviral activity of seven different oligonucleotide analogs.[98] Each oligonucleotide consisted of the identical 12 nucleotide sequence targeted at the *tat* splice acceptor site region. The oligonucleotides were modified at the backbone linkage, the sugar moiety, or both, including phosphorothioate, 2'-*O*-alkyl-ribonucleotide, 2'-OMe-ribonucleotide, and α-anomer. All of the oligonucleotides protected cells against HIV infection with an IC_{50} ranging from >100 μM for the phosphodiester oligonucleotide to 0.25 μM for the phosphorothioate analog. For each oligonucleotide, the control sequence, containing the same modifications, showed similar levels of inhibition. Other groups used different conjugated and modified oligonucleotides. These conjugations included cholesterol groups,[60,109] lipophilic groups,[110,111] poly(L-lysine),[112,113] and immunoliposomes,[114] which are all presumed to increase the stability and uptake of oligonucleotides. In all cases, there was an increase of anti-HIV-1 activity compared with unmodified oligonucleotide. We also studied the anti-HIV activity of self-stabilized phosphorothioate oligonucleotides that have a stem loop structure at the 3' end[41] (see Figure 3). These oligomers displayed more inhibitory effect than the non-self-stabilized oligonucleotides. Hybrid oligonucleotides were also evaluated for antisense activity against HIV.[99] These oligomers contain 2'-OMe ribonucleotide linkages at the 3' and the 5' ends, whereas the central core is of phosphorothioate (see Figure 3). These hybrid oligonucleotides were more active than their phosphorothioate analog. The inhibition of HIV replication was observed at 0.1 μM.

It is believed today that phosphorothioate oligonucleotides act by two mechanisms: a nonspecific inhibition and a sequence-specific inhibition. The nonspecific inhibition may involve many mechanisms. First, the phosphorothioate oligonucleotides may inhibit the reverse transcriptase and stop the transcription of the viral RNA into DNA, which has been demonstrated with purified HIV-reverse transcriptase. Sd(C)28 is the most potent inhibitor of this enzyme.[104,105] Second, the phosphorothioate oligonucleotides may bind to certain proteins such as the T4 receptor CD4 and the viral protein gp120, thereby inhibiting the binding of the virus and its subsequent adsorption.[115–117] In one study,[93] a library of phosphorothioate octanucleotides was screened for anti-HIV activity. One phosphorothioate oligonucleotide, T2G4T2, was found to be the most potent inhibitor of HIV replication. This oligomer forms a parallel-stranded tetrameric guanosine-quartet structure and binds to gp120 at the V3 loop, thereby inhibiting cell-to-cell and virus-to-cell infection.

Some groups used transfection systems to inhibit viral targets.[91,118,119] In these assays, there is no reverse transcription and no viral adsorption. As expected, only the complementary oligonucleotides exhibited inhibition. Although the transient transfection assay provides evidence of specific inhibition, the results obtained are usually difficult to reproduce.

In a model experiment to test the specific effect of phosphorothioate oligonucleotides, different oligonucleotides were added to MOLT-3 cells either simultaneously with the virus or some time after infection.[100] When phosphorothioate oligonucleotides were added to tissue culture simultaneously with the virus, all of the oligonucleotides, including a homo-oligomer, inhibited viral replication. However, at postinfection times, inhibition was observed only for the complementary oligonucleotide. Cells chronically infected with HIV have been used to obtain specific inhibition with oligonucleotides. In this type of study, H9 or MOLT-3 cells were exposed to HIV-1, and then some time later the cells were incubated with the oligonucleotides. Matsukura et al.[84,85] tested the activity of a 28-mer phosphorothioate oligonucleotide anti-rev and compared it to Sd(C)28 and other control oligonucleotides. The anti-rev oligonucleotide inhibited viral production in a dose-dependent manner (IC_{90} at 25 μM), whereas the Sd(C)28 and control oligonucleotides failed to do so, thus confirming the sequence specificity of phosphorothioate oligonucleotides. Kinchington et al.[87] compared the activity of phosphorothioate oligonucleotides complementary to gag, pol, and rev in both acutely and chronically infected cells. Their results showed that oligonucleotides were active in acutely infected cells in the 0.1–2 μM range but had no sequence specificity. In contrast, with chronically infected cells, only the antisense rev compound showed activity at 30 μM, and neither the gag nor the pol antisense compound had a significant effect on HIV replication at 50 μM.

To conclusively demonstrate sequence-specific inhibition of HIV replication, we developed long-term culture experiments.[88,89] In this system, the $CD4^+$ cells were infected with HIV-1 and washed free of virus after a 2-hour incubation. The cells were treated with the phosphorothioate oligonucleotides initially and every 3 or 4 days for several weeks at 1 μM concentration. The efficacy of antiviral activity was monitored by measuring p24. Four 28-mer phosphorothioate oligonucleotides targeted to nucleotides 5970–5997 (rev-1), 5976–6003 (rev-2), 7788–7815 (RRE), and 776–802 (gag-28) in the HIV-1 genome, and 25-mer complementary to nucleotides 778–800 (gag-24) were studied (Figure 7, bottom). As a control, a random 28-mer was used. Treatment of HIV-1-infected cells twice a week with 1 μM concentration of antisense oligonucleotide (gag28) suppressed virus replication for 84 days, the end point of the experiment. This inhibition was still maintained when the concentration of the oligonucleotide was reduced in the middle of the experiment to 0.1 and 0.01 μM.[89] Inhibition was found to be sequence-specific since the random oligonucleotide failed to inhibit HIV replication. Inhibition was also dependent on the target sequence. Rev-1 and rev-2 were targeted to the same gene *rev* but contained overlapping sequences; rev-1 was found to be more active

than rev-2, indicating that minor shifts in the target sequences can change the efficacy of these oligomers. Withdrawal of oligonucleotides from media at day 35 resulted in HIV replication. This indicates that the virus was present in cells but its replication was blocked by the antisense treatment.

Phosphorothioate oligonucleotides were also studied using primary blood mononuclear cells (PBMCs) as target cells. PBMCs were infected with HIV-1, and after 2 hours the cells were washed and incubated with different concentrations of phosphorothioate oligonucleotides. Seven days after infection, viral replication was monitored by p24.[90] Rev and gag oligonucleotides inhibited viral replication in a dose-dependent and sequence-specific manner, whereas the control oligonucleotides failed to show antiviral activity. Ninety percent inhibition was achieved at 1 μM for rev and gag oligonucleotides. The oligomers also inhibited the replication of field isolates and prevented the cytopathic effect of the virus in primary CD4⁺ T cells.[90] Phosphorothioate oligonucleotides also demonstrated potent antiviral activity in primary human macrophages.[90,92,120]

GEM91

GEM91 (gene expression modulator) is being developed as the first antisense therapeutic agent for AIDS (1). Clinical trials in both France and the United States are under way.

GEM91 is complementary to the AUG site of the *gag* region of the HIV-1 genome (Figure 8, top), which is found to be very conserved in HIV-1 strains.[121] GEM91 may inhibit HIV replication by blocking the translation of gag mRNA and may also disrupt the secondary structure of RNA. GEM91 has been extensively studied for its anti-HIV activity. In short-term infection assays GEM91 inhibited viral replication with an IC_{50} of 0.2 μM and an IC_{90} of 0.9 μM[1] and was also effective in long-term experiments at concentrations between 1 and 2 μM. Moreover, GEM91 inhibited HIV replication in PBMCs and macrophages (Figure 8, bottom).[90] Recently the activity of GEM91 was measured using clinical isolates of HIV, which may be the most relevant for clinical situation. This assay is based on primary cells from HIV-1 infected individuals and combines the use of primary cells with primary isolates. GEM91 inhibited HIV-1 replication in all clinical isolates tested to date. This inhibition was paralleled by an increase in the percentage of CD4⁺ T cells. The effectiveness of GEM91 was similar for AZT-sensitive and AZT-resistant virus isolates.

B. Herpes Simplex Virus

Herpes simplex viruses (HSVs) are among the most common infectious agents of humans. There are two different distinct serotypes, HSV-1 and HSV-2. HSV-1 infections occur frequently during childhood and affect most often the mouth, lips, and skin sites above the waist. HSV-2 infections occur most often during adolescence and involve skin sites below the waist, primarily the genitals.[122] The DNAs
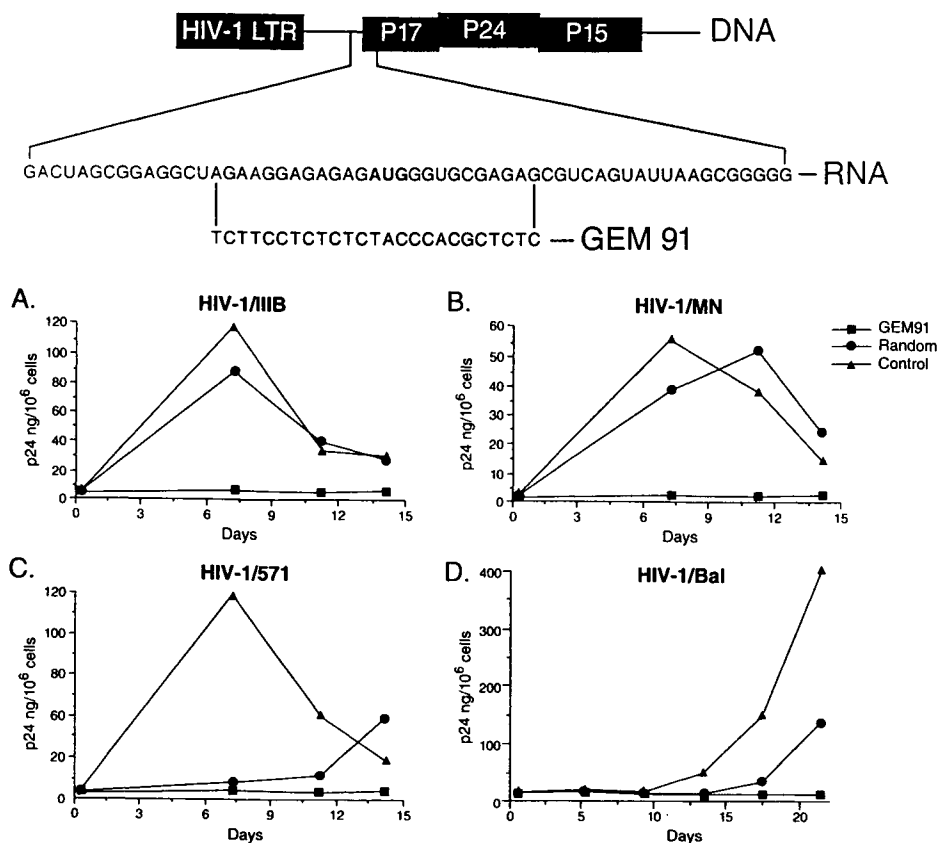
GACUAGCGGAGGCUAGAAGGAGAGAGAUGGGUGCGAGAGCGUCAGUAUUAAGCGGGGG — RNA

TCTTCCTCTCTCTACCCACGCTCTC — GEM 91

Figure 8. Top: Sequence of GEM91 and the targeted region in HIV-1 genome. Bottom: GEM91 blocks the replication of HIV-1/IIIB (**A**), HIV-1/MN (**B**), HIV-1/571 (**C**), HIV-1/BaL in primary human macrophages (**D**). (Reprinted from Ref. 90.)

of HSV-1 and HSV-2 are linear double-stranded molecules with molecular weights of approximately 100 million. About half of the HSV-1 and HSV-2 DNA sequences are homologous. The genome consists of two components, designated L and S, each of which is flanked by inverted repeat (IR) sequences.

Different modified oligonucleotides have been used to inhibit HSV. Phosphodi-ester oligonucleotides complementary to the translation initiation region of HSV-1 Vmw65 RNA were shown to inhibit HSV-1 replication in LTK⁻ cells.[123] Fifty percent inhibition was observed at 150 nM. However, the cells were incubated with the oligonucleotide 18 hours before infection with HSV-1. A 13-mer phosphodiester oligonucleotide in α-anomeric configuration complementary to the splice junction of HSV-1 immediate-early pre-mRNAs 22 and 47 was substituted at the 5′ end with psoralen derivative and added to infected Vero cells.[124] Eighty percent inhibition

was observed at 0.5 μM. In this case, the psoralen derivative required activation by UV irradiation.

Methylphosphonate oligonucleotides have also been evaluated for antisense activity against HSV. A series of short oligonucleotides containing methylphosphonate linkages were targeted to the splice junction of HSV-1 immediate-early pre-mRNAs 4 and 5.[70,72] The target gene plays a regulatory role in HSV replication, and splicing is involved in the control of gene expression. The methylphosphonate oligonucleotides were added at different concentrations to Vero cells at the time of infection with HSV. When the oligonucleotides were added to Vero cells at the same time as the infection by HSV-1, the inhibition was specific and dose dependent. Fifty percent inhibition was observed at 15 μM and 90% inhibition at 100 μM. When the oligonucleotides were added at 1 hour post-infection, however, a five- to tenfold reduction in potency was observed. A psoralen derivative of this oligonucleotide was threefold more potent than the unconjugated compound. Here also, the psoralen derivative required activation by UV irradiation following addition to the infected cells.

A 12-mer methylphosphonate that was active in cell culture was also evaluated for its anti-HSV activity in an infected BALB/c mouse model.[125] When the animals were injected intradermally with a single dose of the oligonucleotide (500 μM) at the time of infection, no efficacy was observed. Similar results were obtained when the animals were injected with 100 μM of the oligonucleotide followed by daily topical applications of 100 μM for 5 days. When this latter treatment was carried out with 500 μM, about 90% decrease in viral replication was observed. The psoralen derivative was tenfold more active when the ear was irradiated for 10 min after each topical application.

Phosphorothioate oligonucleotides have also been evaluated for their anti-HSV activity. A phosphorothioate oligonucleotide complementary to the internal AUG site of UL13 mRNA in HSV-1 reduced HSV-1 infection in Hela cells.[52] The protein encoded by the *UL13* gene has been putatively identified as a phosphotransferase that might be involved in the phosphorylation of viral capsid proteins.[126,127] The cells were incubated with the oligonucleotides 18 hours prior to infection with HSV-1. The phosphorothioate oligonucleotide reduced viral yields by 90% at 4 μM. The same sequence was much less active when used as a phosphodiester or when it contained three phosphorothioate linkages at both the 3′ and the 5′ ends. The effect of phosphorothioate homo-oligonucleotides on the replication of HSV-2 was studied in cell culture.[128] Sd(C)28, which inhibited HSV-2 replication in a dose-dependent manner, was the most potent inhibitor, giving 90% inhibition at 1 μM. The homo-oligomers were found to be more potent inhibitors of HSV-2 than of HSV-1.

The nonspecific inhibition observed with these homo-oligomers could result from at least two different mechanisms: inhibition of the viral DNA polymerase or interference of viral adsorption or penetration. The effect of these homo-oligomers on the DNA polymerase was evaluated in vitro using purified HSV-1 and HSV-2 DNA polymerases.[129] The homo-oligomers inhibited the DNA polymerase activity

in a length-dependent manner, with Sd(C)28 being the most active compound. The inhibition of HSV DNA polymerases by Sd(C)28 was compared to other polymerases such as human DNA polymerase α, β, and γ. The compound showed less inhibition for the human polymerases. This homo-oligomer also inhibits HIV reverse transcriptase.[104] The fact that Sd(C)28 is active against viral DNA polymerase while relatively sparing of mammalian enzymes makes it a good candidate for therapeutic applications. When applied topically in an HSV-1 infected keratitis rabbit model,[5] Sd(C)28 showed a potent and selective inhibition.

We also studied the antiviral activity of self-stabilized phosphorothioate oligonucleotides against HSV-1. The self-stabilized and the non-self-stabilized oligonucleotides were added to infected Vero cells; after 3 days, the plaques were counted. The inhibition was dose dependent for both oligonucleotides, but nevertheless, the self-stabilized oligonucleotide was more active than the non-self-stabilized. The IC_{50} for the non-self-stabilized was >8 μM, whereas for the self-stabilized, it was 5 μM.[130]

C. Influenza Virus

Influenza virus infections are the most important cause of medically attended acute respiratory illness in the world. The impact of these viruses is universal, affecting people of all ages. A long-term vaccination approach has failed, mainly because of the high degree of antigenic variation among influenza viruses. The influenza viral genome consists of eight pieces of negatively stranded RNA, each 500–2000 bases in length. The 3' end of all the influenza viruses' genome pieces consist of the same 13 terminal nucleotides. The 5' ends are to a large degree complementary to the 3' ends, but with some mismatches, and with less strict conservation than the 5' end sequences.

We synthesized 20-mer unmodified oligonucleotides complementary to the 3' end of the eight genome strands and tested them for inhibitory effects on Influenza A and C viruses, grown in tissue culture in MDCK cells.[131,132] These oligonucleotides failed to inhibit viral replication at concentrations up to 80 μM, possibly because of nuclease digestion of these oligonucleotides in the cell culture media. In contrast, phosphorothioate analogs of these oligonucleotides were found to inhibit replication of both influenza A and C viruses, in a dose-dependent and sequence-specific manner. The greatest inhibition was obtained for a negatively stranded 20-mer with the same sequence as the *PB1* gene that is part of the polymerase complex required for transcription and replication of viral RNA. The antiviral effect was found at concentrations as low as 1.25 μM, and 90% inhibition was observed at 15 μM. At this latter concentration, no inhibition was observed for a mismatched oligonucleotide. We also used self-stabilized oligonucleotides and compared their activity to the non-self-stabilized in MDCK cells.[130] The self-stabilized oligonucleotide was 25-fold more potent than the non-self-stabilized. In another study, short oligonucleotides covalently linked to an acridine derivative

were tested.[133] Addition of the acridine-oligonucleotide complementary to the conserved 3' end at concentrations from 50 to 100 µM to MDCK at the time of infection prevented the cytopathic effect of the virus when checked 3 days post-infection. The inhibition was specific since a control acridine-substituted oligonucleotide failed to do so. The replication of influenza virus A in MDCK cells has also been shown to be inhibited by oligonucleotides combined with hydrophobic undecyl residue,[134] which increases the uptake of the oligonucleotide. At 100 µM, the substituted oligonucleotide suppressed virus reproduction and virus-specific protein synthesis, whereas the unmodified and the modified noncomplementary oligonucleotides did not affect virus reproduction.

D. Human Papilloma Virus

Human papilloma viruses (HPVs) are small DNA viruses that infect a wide range of mammals and vertebrates,[135] inducing the hyperproliferation of epithelial cells and causing warts. There are several types and subtypes that infect different species. Certain HPV types, in particular HPV-16 and HPV-18, have been implicated as possible causative agents in the development of cervical cancer.[136] Despite its widespread occurrence as a sexually transmitted disease and its association with malignant disease, no specific antiviral agents for papilloma virus exist.

Antisense oligonucleotides were used to target the papilloma E2, E6, and E7 proteins in cells infected with HPVs. The E2 open reading frame of papilloma virus encodes a family of sequence-specific DNA-binding proteins that regulate transcription of papilloma early genes.[137,138] E6 and E7 genes are required for acquisition and maintenance of a fully transformed phenotype.[139,140]

Storey et al.[141] compared the activity of different phosphorothioate oligonucleotides complementary to different sites within the E6/E7 region of HPV. The effect of these oligonucleotides was tested on CaSki cells, a human cervical carcinoma cell line that contains many integrated copies of the HPV-16 genome. The effect of the oligomers was monitored by measuring cell proliferation by [³H]thymidine uptake. The oligonucleotides that overlapped the E6 and E7 start sites were shown to have the greatest effect on cell proliferation. They inhibited cell proliferation at concentrations of 1–5 µM. Although these oligonucleotides inhibited cell proliferation, this was not accompanied by a concomitant decrease in the targeted proteins. Surprisingly, the same oligonucleotides were able to decrease the cell proliferation of another cell line that lacks the HPV-16 genome, suggesting a nonspecific effect. In contrast, Steele et al.,[142] using similar oligonucleotides targeted to the E6 and E7 genes of HPV-18, showed that the inhibition of cell proliferation is specific. In the oral cancer cell line 1483 and the cervical cancer cell line C4-1, the oligonucleotides inhibited the cell proliferation at 1–5 µM. No inhibition was observed when the oligonucleotides were incubated with cell lines that lack the HPV-18.

The difference between these two studies remains unclear but might depend on the choice of cells that were used as a negative control. The effects of antisense oligonucleotides on HPV infection are difficult to assess because of the lack of a suitable tissue culture system or animal model, in spite of many years of effort. This is thought to be due, in part, to the fastidious requirement for differentiating epithelium by the virus. In addition, papilloma viruses do not encode a viral DNA polymerase, relying instead on host cell DNA replication machinery. In the existing systems, there is no active production of virus.

Cowsert et al.[3] used a cell transfection system in which the E2 protein of bovine papilloma virus (BPV) transactivates the chloramphenicol acetyl transferase (CAT) gene. One phosphorothioate oligonucleotide complementary to the AUG site of E2 inhibited the expression of CAT protein in BPV-transformed C127 cells. The inhibition was sequence dependent by comparison to the activity of random oligonucleotides and was concentration dependent. The IC_{50} was in the range of 50 to 100 nM.

A 20-mer phosphorothioate oligonucleotide (ISIS 2105) complementary to the initiation site of E2 shared by HPV-6 and HPV-11 was tested in assays similar to those used with BPV. This oligonucleotide inhibited the CAT expression in a sequence-specific and dose-dependent manner. The IC_{50} was in the range of 5–7 μM. The authors argue that the difference observed in IC_{50} between BPV and HPV assays can be attributed to the fact that HPV-E2 is being expressed from a strong surrogate promoter, whereas BPV-E2 is expressed from a weak one. Clinical trials for ISIS 2105 are under way.[6]

E. Epstein-Barr Virus

Epstein-Barr virus (EBV) is a member of the herpes group of viruses and has been implicated as having a causal relationship to African Burkitt's lymphoma and nasopharyngeal cancer.[143,144] It has also been linked to other diseases such as chronic mononucleosis and chronic fatigue syndrome.[145,146] Five 28-mer phosphorothioate oligonucleotides were examined for their effect on EBV proliferation in H1 cells that express EBV early antigen and membrane antigen.[147] Five days after the addition of the oligonucleotides to H1 cells, the amount of EBV DNA and virus yield were measured. Each of the five phosphorothioate oligomers inhibited EBV production by over 90% at 1 μM concentration. The inhibition was sequence independent since Sd(C)28 oligonucleotide gave similar levels of inhibition. The mechanism of action appears to be the inhibition of EBV DNA polymerase activity. As described earlier, Sd(C)28 has also been shown to inhibit the DNA polymerase of other viruses such as herpes simplex virus.[129]

In a recent study[148] the EBNA-1 gene, which is required for replication and maintenance of the episomal viral genome in latently infected cells, was targeted using a phosphodiester oligonucleotide. Exposure of EBV-immortalized lymphoblastoid cells to EBNA-1 oligonucleotide partially suppressed the EBNA-1

protein expression. The inhibition was greater than that seen with scrambled sequences or untreated cells.

F. Hepatitis (B and C)

Hepatitis B virus infection can cause a broad spectrum of diseases ranging from asymptomatic infection to fulminant hepatitis. Hepatitis virus B (HBV) is a small circular DNA that is partly single-stranded. Recent studies suggest that the mechanism of replication involves reverse transcription.[149] Hepatitis C virus (HCV) is the major causative agent of post-transfusion hepatitis, and persistent HCV infections often progress to chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The genome of HCV is a positive-sense, single-stranded linear RNA.[150,151]

The effect of a series of antisense oligonucleotides on the expression of the surface antigen (HB_sAg) of HBV was examined using hepatocellular carcinoma cells that contain integrated HBV genomes.[152] The oligonucleotides directed at the cap site and the initiation region of the HB_sAg gene were found to be highly effective. More than 90% inhibition was observed for phosphodiester oligonucleotides at 17 μM and 5 μM for phosphorothioate analog. In another study, phosphorothioate oligonucleotides complementary to the polyadenylation signal of HBV were incubated with the HepG2 cell line, which is permanently transfected with HBV.[153] The phosphorothioate oligonucleotide gave specific inhibition of HBV surface antigen at 50 μM. For HCV, the effect of oligonucleotides was measured in a cell free system using rabbit reticulocytes.[154] Oligonucleotides that cover the initiation codon and sequences immediately downstream blocked HCV translation in vitro.

Although many attempts have been made to cultivate human hepatitis viruses in cell culture systems and to establish small animal models of disease, none were successful. A major achievement facilitated the evaluation of therapeutic strategies to terminate HBV infection: the discovery of animal viruses closely related to HBV, e.g., duck hepatitis B virus (DHBV). DHBV-infected primary duck hepatocyte cultures were used to assess the antiviral activity of nine phosphorothioate antisense oligonucleotides.[155] The most effective antisense oligonucleotide was directed against the 5' region of the pre-S gene and resulted in complete inhibition of viral replication at 1.5 μM. The corresponding sense oligonucleotide did not significantly affect viral replication. The effective oligonucleotide was then evaluated for its antiviral activity in animals. Two DHBV-negative Pekin ducklings were treated with the oligonucleotide at a concentration of 20 μg/g body weight. Twelve hours later the animals were infected intravenously with DHBV DNA positive serum. Animals were treated daily by an intravenous injection of the oligonucleotide until the animals were sacrificed (day 12). There was an absence of viral DNA and pre-S proteins, indicating successful prevention of the infection by pretreatment of animals with the complementary oligonucleotide. No major inhibition was observed with the corresponding sense oligonucleotide.

G. Cytomegalovirus

Human cytomegalovirus (HCMV) is a ubiquitous herpes virus that causes mild or subclinical disease in immunocompetent adults but can cause severe complications in immunosuppressed people. CMV is now the most common opportunistic ocular infection associated with AIDS, occurring in 15 to 40% of all patients. Untreated, it progresses rapidly to blindness. CMV is the largest member of the human herpes virus family. The genome is a double-stranded DNA of approximately 240 kilobases.

Different phosphorothioate oligonucleotides complementary to the major immediate-early (IE) transcriptional unit of HCMV or to HCMV DNA polymerase have been tested for their antiviral activity in infected NHDF cells from foreskin.[2] One phosphorothioate oligonucleotide (ISIS 2922) complementary to the mRNA encoding IE2 protein showed potent antiviral activity and was more potent than ganciclovir. In this experiment, however, the oligonucleotide was added to cells prior to infection with HCMV. The antiviral activity of ISIS 2922 was affected by the serum concentration in tissue culture media, decreasing with increasing concentrations of the serum. This compound is in clinical trials for the treatment of AIDS patients with refractory CMV retinitis.[6]

Smith and Pari used a 20-mer phosphorothioate oligonucleotide (UL36ANTI) complementary to the intron-exon boundary of HCMV genes *UL36* and *UL37*.[156] These genes are required for HCMV origin-dependent DNA replication. Human foreskin fibroblasts were incubated with the oligonucleotide 15 hours prior to infection, and DNA replication was measured by Southern analysis. UL36ANTI inhibited HCMV DNA replication at concentrations as low as 0.08 μM. Northern blot analysis showed that treatment with UL36ANTI decreased steady-state UL36 mRNA to undetectable levels, whereas RNA levels of a second immediate-early gene (IE2) were unaffected. Base substitutions that result in base pair mismatches showed lesser degrees of activity, indicating a sequence-specific mechanism. Antiviral activity was not observed with the sense oligonucleotide and other control phosphorothioate oligonucleotides. UL36ANTI also inhibited DNA replication of ganciclovir- and PFA-resistant HCMV strains. As with ISIS 2922, the antiviral activity of UL36ANTI was affected by the concentration of the serum.

H. Vesicular Stomatitis Virus

Vesicular stomatitis is a disease of cattle, horses, and swine. The vesicular stomatitis virus (VSV) genome consists of a single RNA negative strand of about 11,000 nucleotides, encoding five proteins, N, NS, M, G, and L.[157,158] The genomic RNA has a negative polarity and it serves as a template for transcription of viral RNAs and for replication through (–)/(+) intermediates.

Short 9 mer methylphosphonate oligonucleotides complementary to the initiation codon regions of N, NS, and G proteins were tested for their ability to inhibit VSV

replication in mouse L cells.[159] L929 cells were first incubated with 0–150 µM of the oligonucleotide before infection with VSV (m.o.i. of 5). Six or twenty-four hours post-infection, the titer of the virus was determined. The synthesis of viral proteins was also checked after pulse-labeling with [^{35}S]methionine. An oligonucleotide complementary to the G mRNA was found to be the most active. The inhibition of virus titer was observed at high concentrations of 100 µM and above, even though the oligonucleotide was incubated with the cells 16 hours prior to infection. The requirement of high concentrations of methylphosphonate oligonucleotide to inhibit VSV replication is probably the result of the fact that these oligonucleotides do not activate RNaseH.

In another study, short phosphodiester oligonucleotides coupled at the 3′ end to poly-L-lysine were targeted to VSV N protein.[160,161] This chemical coupling was used to increase the cellular uptake of the oligonucleotides. Infected mouse L929 cells were incubated with these oligonucleotides and the virus titer was measured 20 hours after infection. When the oligo-conjugate was targeted to the coding sequence of N protein, no effect on virus production was observed, but when the oligo-conjugate was targeted to the initiation codon of the N protein, a drastic inhibition of the synthesis of VSV proteins was observed at concentrations below 1 µM. Noncomplementary oligonucleotides had no effect. Similarly, when the oligonucleotides were not linked to poly-L-lysine, no inhibition was observed, even at high concentrations (50 µM). Moreover, α-anomeric oligonucleotides conjugated to poly-L-lysine failed to inhibit virus production.[162] This observation indirectly argues for the implication of the RNase H in the mechanism of inhibition by oligonucleotides. Even though poly-L-lysine increases the cell uptake and the stability of the oligonucleotide, it is toxic to the cells, and high concentrations of poly-L-lysine can lead to inhibition of cell growth.

The same group used a different approach for delivery of oligonucleotides across the cell membrane using antibody-targeted liposome.[163] The liposome-encapsulated antisense oligomers reduced virus production in vitro 100 times more efficiently than the nonencapsulated oligomer. In another study,[62] a phospholipid was attached at the 5′ end of a phosphodiester oligonucleotide to increase uptake in L929 cells. Although the unmodified oligonucleotide was inactive in VSV antiviral assay at 200 µM, the attachment of a lipid to the oligomer led to more than 90% inhibition at 150 µM.

I. Rous Sarcoma Virus

In 1978 Zamecnik and Stephenson used a 13-mer phosphodiester oligonucleotide complementary to the 3′ and 5′ reiterated terminal sequences of Rous sarcoma virus RNA to inhibit viral replication in infected chicken fibroblasts.[7,8] This oligonucleotide showed inhibition at 2 µM, and the inhibition effect was much greater at a lower multiplicity of infection. The oligonucleotide was then protected at both

the 3' and 5' ends with a chemical group to increase its nuclease resistance in the cell culture media, which increased its potency.

VIII. PHARMACOKINETICS OF ANTISENSE OLIGONUCLEOTIDES

The potential utility of antisense oligonucleotides for the treatment of viral infections and other diseases will depend on whether effective concentrations of oligonucleotide reach the target site of action in vivo. We and others have studied the pharmacokinetics and biodistribution of antisense oligonucleotides in animals.[17,26,31,32,164,165] Phosphodiester oligonucleotide injected intravenously in monkeys and mice are rapidly degraded in the plasma,[17,18] which is within about 5 minutes. No intact oligonucleotide could be detected after 15 minutes post-dosing. This short half-life of phosphodiester oligonucleotides limits their use as therapeutic agents. The methylphosphonate oligonucleotides also have a short elimination half-life of about 17 minutes in mice when injected intravenously. More than 70% of the oligonucleotide was excreted in urine in the first 2 hours. To be used as systemic drugs, methylphosphonate oligonucleotides will require frequent dosing. Nevertheless, these oligomers may be useful in topical applications because of their lipophilic properties.

Administration of a single dose of phosphorothioate oligonucleotide in animals by the intravenous route revealed a biphasic plasma elimination. The initial short half-life, ranging from 0.53 to 0.83 hours, represents distribution out of the plasma compartment, and a second long half-life (35 to 50 hours) represents the elimination from the body (Figure 9). The elimination half-life was similar if the oligonucleotide was administered subcutaneously. The plasma clearance of the phosphorothioate oligonucleotide was not affected by the oligonucleotide length or the base sequence. Phosphorothioate oligonucleotides were distributed in most of the organs of rats and mice. The liver and kidney were the two organs with the highest uptake of oligonucleotides.[26] Brain had the lowest concentration. The phosphorothioate oligonucleotides were primarily excreted in urine, with up to 30% excreted in the first 24 hours.[26] Phosphorothioate oligonucleotides were stable in most of the organs except for liver and kidney, where 50% degradation was observed at 48 hours post-dosing. Repeated, daily intravenous injections of a 25-mer phosphorothioate oligonucleotide into rats showed that the concentrations in the plasma remained in a steady state during the 8-day administration.

The phosphorothioate oligonucleotides have favorable absorption and distribution kinetics and sufficient in vivo stability to be used as therapeutic agents for viral infections and other diseases. The relatively long elimination half-life in animal plasma suggests that infrequent administration could be used to maintain a therapeutically effective concentration of phosphorothioate oligonucleotides.
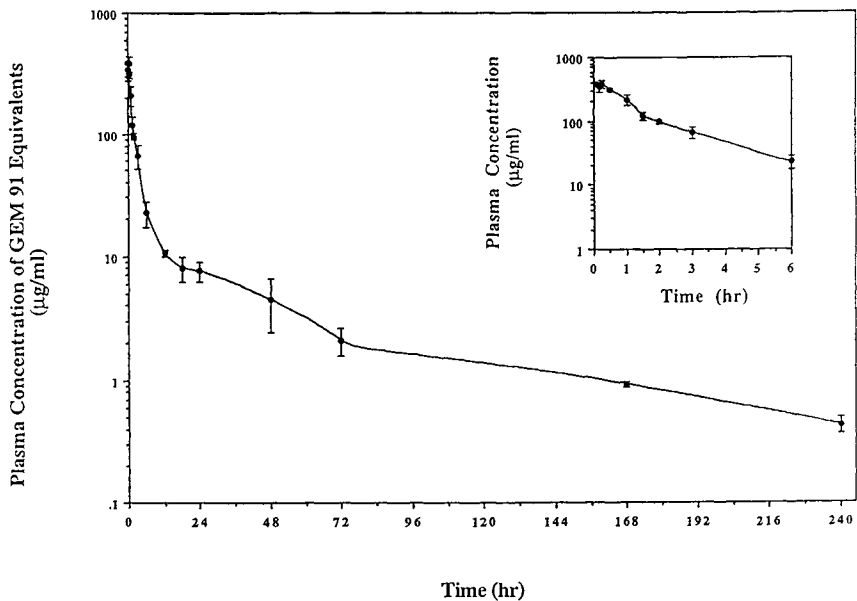
Figure 9. Pharmacokinetics of GEM91 in rats. Plasma concentration time course of GEM91-derived radioactivity. ^{35}S-labeled GEM91 was injected into rats intravenously at a dose of 30 mg/kg. Plasma concentration was expressed as micrograms of GEM91 equivalents per milliliter (mean ± SD). Inset, expanded time course over initial 6 hours. (Reprinted from Ref 165.)

Clinical trials using antisense oligonucleotide therapy for papilloma viruses, cytomegalovirus, and HIV have recently been initiated, all using phosphorothioate oligonucleotides. In the case of papillomavirus, the oligonucleotide is injected intradermally at the site of genital warts. For treatment of cytomegalovirus, the oligonucleotide is injected intravitreally. For HIV, we have administered a single dose of a 25-mer ^{35}S-labeled phosphorothioate oligonucleotide (GEM91) into six HIV-1-infected individuals by 2-hour intravenous infusion at a dose of 0.1 mg/kg to assesses the plasma clearance profile and urinary excretion. The plasma disappearance curve was described by the sum of two exponentials with mean half-lives of 0.18 and 26.7 hours based on radioactivity levels. Urinary excretion represented the major pathway of elimination of oligonucleotide. On the basis of radioactivity levels, about 49% of the administered dose was excreted within 24 hours and about 70% within 96 hours of oligonucleotide administration.[166] Analysis of the extracted radioactivity in plasma showed both intact and degraded forms of phosphorothioate oligonucleotide. In urine, however, all of the radioactivity was associated with the degraded form of the oligonucleotide.

IX. CONCLUSION

In this chapter we have reviewed antiviral activities of antisense oligonucleotides. Several significant points emerge from this overview. First, the rapid progress in antisense technology during the last 10 years has yielded encouraging results, in both basic science and therapeutic applications. Second, cell culture and animal experiments have provided evidence that oligonucleotides have inhibitory effects on viral infectivity, function, and replication. Third, antisense technology is broadly applicable to antiviral diseases and to other illnesses.

Several characteristics of oligonucleotides are essential for their antiviral activity, including the ability to be taken up by cells, to resist degradation by nucleases, and to hybridize with the target structure. The ability to activate RNase H is also essential. The mechanism by which oligonucleotides inhibit viral replication remains to be elucidated. It seems to involve both sequence-specific and non-sequence-specific mechanisms. Further studies on the precise mechanism of antisense activity could give clues to the design of more potent oligonucleotides. In the meantime, the development of the most active first-generation oligonucleotides, the phosphorothioates, is proceeding. These oligomers show favorable pharmacologic, toxicologic, and pharmacokinetic properties, therefore displaying the potential of antiviral activity. Clinical trials of antisense oligonucleotides are under way to treat diseases caused by papilloma virus, human immunodeficiency virus, cytomegalovirus, and cancer. Several other candidates await further development. The success of these oligonucleotides as chemotherapeutic agents will also depend on the ultimate cost. Large-scale applications and new methodologies are being implemented to increase the yield and reduce the cost of oligonucleotides. Because of the progress made to date and the tremendous potential of antisense oligonucleotides, it is reasonable to state that we can expect to see regular use of oligonucleotides as therapeutic drugs in the coming years.

ACKNOWLEDGMENTS

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DESIGN AND SYNTHESIS OF
S-ADENOSYLHOMOCYSTEINE
HYDROLASE INHIBITORS
AS BROAD-SPECTRUM
ANTIVIRAL AGENTS

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I. INTRODUCTION

S-Adenosyl-L-methionine (AdoMet) serves as a methyl donor for a variety of cellular enzymatic transmethylation reactions.[1] In AdoMet-dependent transmethylation reactions, the methyl group from AdoMet is transferred to various acceptor molecules such as proteins, nucleic acids, phospholipids, and small molecules by specific methyltransferases.[2–12] S-Adenosyl-L-homocysteine (AdoHcy) is a product of all AdoMet-dependent transmethylation reactions.[13] In eukaryotes, the only pathway known for the catabolism of AdoHcy is its hydrolysis to adenosine (Ado) and homocysteine (Hcy) by AdoHcy hydrolase (EC 3.3.1.1).[14–16] In recent years, AdoHcy hydrolase has become an attractive target for drug design because inhibitors of this enzyme have been shown to exhibit antiviral,[17–19] antiparasitic,[20,21] anti-arthritic,[22] and immunosuppressive effects.[23,24]

Metabolism of AdoHcy by rat liver AdoHcy hydrolase was first described in 1959 by de la Haba and Cantoni.[25] The enzyme was shown to catalyze the reversible hydrolysis of AdoHcy to Ado and Hcy, with the equilibrium favoring the synthetic direction. However, under physiological conditions, the reaction is pulled in the hydrolytic direction by the enzymatic removal of both Ado and Hcy.[25] The mechanism by which AdoHcy hydrolase catalyzes this reaction was elucidated by Palmer and Abeles.[26] Mammalian AdoHcy hydrolase, which is a homotetramer, contains tightly (but not covalently) bound NAD^+ (approximately one NAD^+ per subunit). The first step in the enzymatic reaction involves oxidation of the 3′-hydroxyl group of AdoHcy to form 3′-keto-AdoHcy (oxidative activity), resulting in the conversion of NAD^+ to NADH. The 3′-keto group increases the acidity of the C-4′ proton, allowing for abstraction of this proton by a base in the active site of the enzyme.

Subsequently, β-elimination of Hcy results in the formation of the intermediate 3'-keto-4',5'-didehydro-5'-deoxyAdo. Addition of water (hydrolytic activity) to the 5' position of this intermediate affords 3'-keto-Ado, which is then reduced by the enzyme-bound NADH, resulting in the formation of Ado and regenerating the NAD⁺ form of the enzyme. In this mechanism, the hydrolytic activity (e.g., elimination of Hcy from 3'-keto-AdoHcy or addition of water to 3'-keto-4',5'-didehydro-5'-deoxyAdo) of the enzyme is dependent on its oxidative activity.

The primary structures of AdoHcy hydrolase from seven different sources (human placental,[27] rat liver,[28] *Dictyostelium discoideum*,[29,30] *Leishmania donovani*,[21] *Petroselinum crispum*,[31] the nematode *Caenorhabditis elegans*,[32] and bacterium *Rhodobacter capsulatus*[33]) have been deduced from the nucleotide sequences of their encoding cDNAs. The amino acid sequences for human placental and rat liver enzymes exhibit 97% homology. All seven cloned AdoHcy hydrolases have a conserved NAD⁺ binding site with a sequence of GYGDVGK. However, the tertiary structure of AdoHcy hydrolase is at present unknown, and crystals of the quality necessary for X-ray diffraction have proved elusive.

One of the physiological roles of AdoHcy hydrolase is to regulate AdoMet-dependent biological methylation reactions.[14] AdoHcy is a potent competitive inhibitor of all AdoMet-dependent methyltransferase.[14,34] Inhibition of cellular AdoHcy hydrolase results in an intracellular accumulation of AdoHcy, causing a significant increase in the intracellular AdoHcy/AdoMet ratio and the subsequent inhibition of AdoMet-dependent methylations.[35–40] Since 1978, when De Clercq et al.[41,42] reported that the AdoHcy hydrolase inhibitor (S)-9-(2,3-dihydroxypropyl)adenine [(S)-DHPA)] was a broad-spectrum antiviral agent, considerable interest has been directed toward this enzyme as a target for the design of antiviral agents. In the past 15 years, significant progress has been made in designing inhibitors of the enzyme and in elucidating its mechanism of catalysis and its physiological roles. In this article, we do not intend to provide a comprehensive review of the vast primary literature concerning AdoHcy hydrolase and its inhibitors (see references 14, 43, and 44 for reviews). Instead, we will focus on the rationale for selecting AdoHcy hydrolase as a target for the design of antiviral agents, how selective inhibitors with improved antiviral effectiveness were designed, how these inhibitors inactivate the enzyme, and how this enzyme regulates crucial biological processes (e.g., viral replication). In addition, we will describe recent relevant nucleoside chemistry that has arisen from those laboratories involved in the design and synthesis of AdoHcy hydrolase inhibitors.

II. RATIONALE FOR SELECTING S-ADENOSYLHOMOCYSTEINE HYDROLASE AS A TARGET FOR ANTIVIRAL CHEMOTHERAPY

In recent years, advances in the molecular biology of viral replication have led to more rational approaches to the design of antiviral chemotherapeutic agents. Based

on these advances in the molecular biology of viral replication, the following steps are potential targets for the design of antiviral agents: (1) entry and uncoating; (2) transcription of viral mRNA (or cDNA in the case of the retroviruses); (3) replication of viral DNA or RNA; (4) viral mRNA methylation or capping; (5) translation of viral mRNA into protein; (6) post-translational cleavage of protein; (7) virus assembly; and (8) viral envelope synthesis and processing.[45-52]

Our laboratories, along with several others, have focused on the methylation of the capped structure on viral mRNA as one approach to the design of antiviral agents. This strategy is based on the observation that many viruses require 5'-capped, methylated structures on their mRNA for efficient translation of viral proteins.[51,53,54] All capped, methylated structures consist of a N-7-methyl guanosine residue linked at the 5'-hydroxyl group to the 5' end of the mRNA strand by a triphosphate linkage. Most 5'-capped, methylated structures also contain a methyl group on the 2'-hydroxy group of the penultimate nucleotide having a general structure of $m^7G(5')ppp(5')N^m$, as shown in Figure 1. This 5'-capped, methylated structure has been shown to protect mRNA from 5'-end nuclease digestion, thereby enhancing its stability in the cytoplasm.[55] In addition, methylation of the capped structures increases the affinity for ribosome binding to the 5' end of the mRNA during formation of the translational initiation complex.[56] Studies of several viral replication systems have revealed a direct relationship between the presence of 5'-cap structures and completed viral mRNA translation.[57,58] Both capping and methylation are enzymatically catalyzed processes. A number of viruses have the capping enzyme (RNA guanyltransferase) and the methylation enzymes (mRNA guanine-7 and nucleoside-2' methyltransferases) within the virion (e.g., vaccinia virus,[59] reovirus,[60] vesicular stomatitis virus,[61] Newcastle disease virus,[62] and polyhedrosis viruses[63]). In contrast, other viruses (e.g., herpes) are presumed to use host cell capping enzymes.[64] Because uncapped or undermethylated viral mRNA is less effectively translated into viral proteins,[65] these 5'-capping and methylation
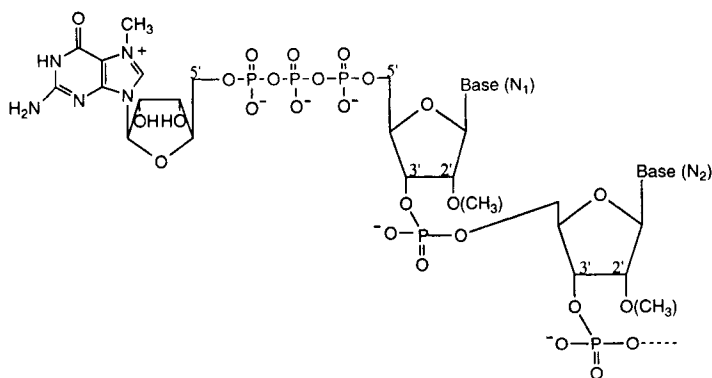

Figure 1. Structure of mRNA 5'-terminal cap.

reactions (e.g., through the inhibition of guanyltransferase, mRNA guanine-7 methyltransferase, or nucleoside-2' methyltransferase) could conceivably lead to inhibition of viral replication.

Since AdoMet was described by Cantoni et al. in 1954,[13] it has been known to be a ubiquitous methyl donor in both eukaryotic and prokaryotic organisms.[36,57,66] AdoMet-dependent transmethylations are involved in the modification of not only small molecules such as histamines, catecholamines, and phospholipids, but also macromolecules such as proteins and nucleic acids (DNA and RNA).[38] It has been shown that viral coded methyltransferases are also AdoMet-dependent.[67–70] For example, both guanine-7 methyltransferase and nucleoside-2' methyltransferase coded by vaccinia virus require AdoMet as a methyl donor.[67–70] The centrality of AdoMet in biological methylation reactions is illustrated in Scheme 1. However, in addition to AdoMet and AdoMet-dependent methyltransferase, effective biological methylations are also dependent on methionine adenosyltransferase (EC 2.5.1.6), which catalyzes the biosynthesis of AdoMet, and AdoHcy hydrolase (or AdoHcy nucleosidase), which catalyzes the metabolism of AdoHcy, the product inhibitor of all AdoMet-dependent methyltransferases. Based on Scheme 1, one might envisage at least three approaches to the design of inhibitors of AdoMet-dependent methyltransferases. These approaches would include inhibitors that function directly on a particular methyltransferase (e.g., analogs of either the methyl acceptor substrate or the methylated product, AdoHcy or AdoMet) or inhibitors that function indirectly by initially inhibiting AdoMet biosynthesis (e.g., methionine adenosyltransferase) or by inhibiting AdoHcy metabolism (e.g., AdoHcy hydrolase, AdoHcy nucleosidase [prokaryotic cells]).

The approach of using analogs of the methyl acceptor substrate or the methylated products has been extensively exploited in the design of inhibitors of "small molecule" methyltransferases (e.g., catechol *O*-methyltransferase, phenylethanolamine *N*-methyltransferase, histamine *N*-methyltransferase, hydroxyindole *O*-methyltransferase, and indolethylamine *N*-methyltransferase).[71–78] This approach has not been used for the design of macromolecule methylase inhibitors. The reasons probably include the difficulty in synthesis of such compounds, the poten-
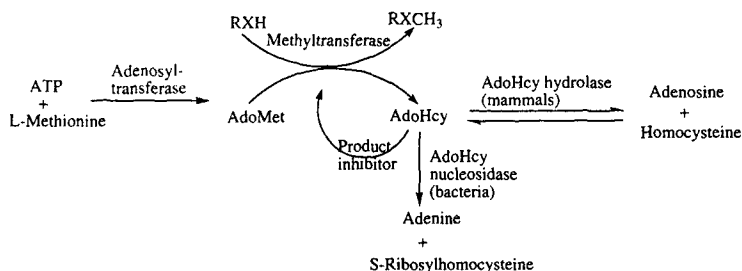
Scheme 1. AdoMet-dependent methyltransferase and AdoHcy metabolism.

tial problems with their cellular transport and metabolism, and the extremely high substrate specificity exhibited by different AdoMet-dependent methyltransferases. Each of the AdoMet-dependent methyltransferases has its own requirements for a methyl acceptor, and each utilizes only a single methyl acceptor substrate or a limited number of structurally related molecules. Analogs of AdoMet, a methyl donor, have also been examined as possible inhibitors of AdoMet-dependent methyltransferases. The enzymatic preparation of such analogs has been somewhat hindered because of the high specificity of adenosyltransferase,[79,80] whereas many others have been prepared by chemical procedures.[72,81,82] In general, the analogs of AdoMet have exhibited poor substrate and/or inhibitor properties for methyltransferases.[72,81] These results indicated that the enzymes have a very high specificity for the structural features of AdoMet. This disadvantage and the problems associated with cellular transport of these compounds have diminished interest in this approach to inhibiting methyltransferases. However, in recent years, significant advances have been made in our understanding of the mechanism by which AdoMet donates the methyl group in enzyme-catalyzed reactions. Specifically, these advances include the elucidation of the absolute configuration of the sulfonium center of AdoMet,[83] the characterization of the mechanism of enzymatic methyl transfer from AdoMet,[84–87] the determination of the stereochemistry of enzymatic methyl transfer from AdoMet,[88] and the characterization of the chemical properties of AdoMet.[89] The availability of this information should now permit the rational design of transition-state-type inhibitors of methyltransferases. Transition-state analogs might provide the desired specificity that is lacking in AdoMet analogs.

A second approach that has been explored in an effort to inhibit AdoMet-dependent methyltransferases is that of altering the activity of methionine adenosyltransferases.[90,91] The result of inhibiting this enzyme would be to decrease intracellular levels of AdoMet, thereby inhibiting all AdoMet-dependent enzymes. Numerous analogs of L-methionine have been prepared and evaluated as inhibitors of methionine adenosyltransferase.[90–95] Administration of some of the most potent methionine adenosyltransferase inhibitors to rodents in vivo resulted in the expected accumulation of L-methionine and depression in the levels of AdoMet in several tissues examined.[96] The apparent disadvantages of this approach include (1) the high structural specificity of methionine adenosyltransferase for L-methionine, resulting in poor inhibitory activity of methionine analogs, and (2) the fact that inhibition of this biosynthetic enzyme will result in a general inhibitory effect on all AdoMet-dependent methyltransferases, as well as polyamine biosynthesis.

Another approach to inhibiting AdoMet-dependent methyltransferases, which has attracted considerable attention and proved quite successful in vivo, is to focus on AdoHcy hydrolase as a primary target.[49,97] AdoHcy is a competitive inhibitor of AdoMet-dependent methyltransferases. The rate of cellular methylation is regulated by existing intracellular ratios of AdoHcy/AdoMet.[34,98] Inhibition of AdoHcy hydrolase results in the intracellular accumulation of AdoHcy, causing a significant

increase in the intracellular AdoHcy/AdoMet ratio and subsequent inhibition of AdoMet-dependent methylation reactions essential for viral replication.[36,65,99–101]

De Clercq and Cools have demonstrated a strong relationship between the log IC_{50} values (the concentration which inhibits viral replication by 50%, against five viruses: vesicular stomatitis, vaccinia, measles, reo, and rota) for a series of AdoHcy hydrolase inhibitors including 3-deazaadenosine (C^3-Ado) and neplanocin A (NpcA), and their log K_i values for inhibition of murine L929 cell or beef liver AdoHcy hydrolase.[43,102,103] In addition, Borchardt and co-workers have correlated the antiviral activities of AdoHcy hydrolase inhibitors with their ability to elevate the cellular levels of AdoHcy.[35,37,100] In recent years, through our increased understanding of the mechanism of the enzyme inactivation and the cellular metabolism of AdoHcy hydrolase inhibitors, more potent and more specific inhibitors of the enzyme have been designed. For example, NpcA analogs including 9-(*trans*-2′-*trans*-3′-dihydroxycyclopent-4′-enyl)adenine (DHCeA) and 9-(*trans*-2′-*trans*-3′-dihydroxycyclopentanyl)adenine (DHCaA) retained inhibitory activity toward cellular AdoHcy hydrolase but were devoid of substrate properties for other cellular enzymes such as Ado kinase and Ado deaminase. This increase in specificity for AdoHcy hydrolase significantly reduced cytotoxicity.[18,19,104–106] These results suggested that rational drug design can be used to identify even more potent and more specific AdoHcy hydrolase inhibitors, which might have clinical use as antiviral agents.

The antiviral activity spectrum of AdoHcy hydrolase inhibitors is unique in that it includes (i) some DNA viruses, such as vaccinia virus, African swine fever virus, and human cytomegalovirus; (ii) (–) RNA viruses, such as the rhabdo (rabies, vesicular somatitis, and infectious hematopoietic necrosis virus) and paramyxo (parainfluenza, measles) viruses; and (iii) (±) RNA viruses (reoviridae: reo- and rotavirus, and infectious pancreatic necrosis virus of fish). In contrast, other DNA viruses (herpetoviridae: herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus) and (+) RNA viruses (picornaviridae: entero (polio, Coxsackie, Echo) and rinoviruses; togaviridae: Sindbis, Semliki forest, tick-bone encephalitis virus), but not the plant (+) RNA viruses (i.e. potex-, poty-, and tymovirus), are much less sensitive or virtually resistant to the inhibitors.[17,41–43,97,102,106–115] The wide range of antiviral activity of AdoHcy hydrolase inhibitors again indicates that AdoHcy hydrolase inhibition is the common mechanism of action.

Broad-spectrum antiviral drugs offer many advantages over narrow-spectrum agents. It is often difficult in clinical diagnoses to identify a viral pathogen in a short time. Results often arrive too late for the choice of a specific antiviral drug. Quite often, immediate action is necessary to prevent the condition of the patient from worsening. Especially in acute infectious, viral chemotherapy must start as soon as the patient presents clinical symptoms. Thus, the development of broad-spectrum antiviral drugs is highly desired.

Since AdoHcy hydrolase is a normal cellular enzyme, inhibition of this enzyme could inevitably cause unwanted cytotoxicity. A total shutdown of AdoMet-dependent methylation reactions would undoubtedly lead to cell death, but apparently partial inhibition can be beneficial in suppressing viral replication. The current dogma does have its exceptions; interference with normal cellular metabolism does not preclude a clinically useful antiviral agent. In fact, to maintain antiviral activity but with reduced cytotoxicity, several approaches have been tried and some have been successful, including (i) partial inhibition or short-term inhibition; (ii) low inhibitor concentrations that do not produce cellular toxicity but perturbate viral replication[19,116]; (iii) synergistic effects with other inhibitors such as ribavirin, an inhibitor of mRNA cap structure formation, or L-cis-AMB, an inhibitor of AdoMet biosynthesis[117,118]; and (iv) design of AdoHcy hydrolase inhibitors that are sufficiently selective to exhibit functions specific to the virus-infected cells with acceptable toxic effects on uninfected cells. It is these possibilities that make AdoHcy hydrolase inhibitors so attractive as broad-spectrum antiviral agents.

III. S-ADENOSYLHOMOCYSTEINE HYDROLASE INHIBITORS—DESIGN, MECHANISM, ANTIVIRAL ACTIVITY, AND CYTOTOXICITY

In the past 15 years, numerous AdoHcy hydrolase inhibitors, both naturally occurring and synthetic compounds, have been identified. These inhibitors can be divided into three groups based on the history of their discovery. Inhibitors from each of the three groups have their unique structural features and biochemical properties. From the first to the third generations, the general trend has been to generate more potent and more specific inhibitors of AdoHcy hydrolase. The evolution of AdoHcy hydrolase inhibitors in the past 15 years reflects our increased understanding of the biochemical properties and mechanism of catalysis of AdoHcy hydrolase and its regulatory role in cellular metabolism.

A. First-Generation Inhibitors

When AdoHcy hydrolase became an attractive target for the design of antiviral agents in the late 1970s and early 1980s, a variety of inhibitors of this enzyme were identified, constituting what we refer to as the first generation of inhibitors.[38,102] Most of these compounds were naturally occurring carbocyclic Ado analogs (e.g., NpcA and aristeromycin (Ari)) or synthetic cyclic or acyclic Ado analogs ((S)-DHPA,[41,42,119,120] D-eritadenine,[17,120-122] (R,S)-3-adenine-9-yl-2-hydroxypropanoic acid ((R,S)-AHPA),[123,124] Ado dialdehyde,[40,125] and C³-Ado).[126-130]

A common biochemical characteristic of the compounds shown in Figure 2 is that they are all inhibitors of AdoHcy hydrolase, with K_i values ranging from 1 nM to 4 μM. (S)-DHPA, an aliphatic nucleoside analog, is reported to be a reversible inhibitor of AdoHcy hydrolase, with a K_i value of 3.5 nM toward rat liver
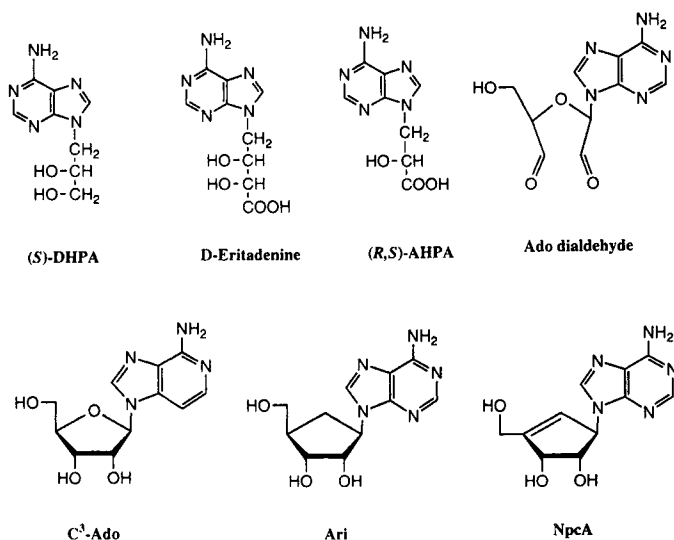
Figure 2. First-generation AdoHcy hydrolase inhibitors.

hydrolase,[43,119] whereas the carboxylic acid derivative D-eritadenine, a naturally occurring compound, is reported to inhibit the enzyme irreversibly ($K_i = 3$ nM).[43,120] Like D-eritadenine, (R,S)-AHPA, which is a monohydroxyalkanoic acid derivative, was also an irreversible inhibitor and showed a K_i value of 73 nM against beef liver AdoHcy hydrolase.[43,102] Among the acyclic compounds, D-eritadenine is the most potent inhibitor of AdoHcy hydrolase. Ado dialdehyde, formed by periodate-oxidation of Ado, structurally resembles the proposed intermediate (3′-keto-4′,5′-didehydro-5′-deoxyAdo) in the catalytic cycle of AdoHcy hydrolase.[26] Ado dial-dehyde has been shown to be a very potent inhibitor of AdoHcy hydrolase, with K_i values ranging from 2 to 4 nM, depending on the assay system.[103,131] Inactivation of AdoHcy hydrolase by Ado dialdehyde is irreversible, and the inhibitor becomes tightly bound to the enzyme, with a stoichiometry of 2 to 4 mol inhibitor per enzyme tetramer.[131,132] Ado analogs with ribosyl or carbocyclic moieties were also effective inhibitors. C^3-Ado was reported[133] to be a reversible, competitive inhibitor of bovine liver AdoHcy hydrolase with a K_i value of 4 μM.[130] Ari, a naturally occurring carbocyclic Ado analog, is also reported to be a reversible, competitive inhibitor of AdoHcy hydrolase.[130] The inhibitory constant (K_i) was determined to be 5 nM for both beef liver and lupin seed AdoHcy hydrolases.[130,134] In contrast, another naturally occurring carbocyclic Ado analog, NpcA, is an irreversible, tightly binding inhibitor of AdoHcy hydrolase, with a K_i value of 8.4 nM for bovine liver enzyme.[39] The inactivation of AdoHcy hydrolase by NpcA is both time- and concentration-dependent. The mechanism of NpcA-induced inactivation of Ado-

Hcy hydrolase involves reduction of the enzyme-bound NAD^+ to NADH with simultaneous oxidation of NpcA to 3'-keto-NpcA, which has been isolated from the inactivated AdoHcy hydrolase under mild denaturing conditions.[135,136] Hence, NpcA inactivates the hydrolase by a "cofactor depletion" mechanism, converting the NAD^+ cofactor to its inactive form (NADH). We refer to compounds that inactivate the enzyme in this way as type I mechanism-based inhibitors (i.e., compounds causing irreversible cofactor depletion but not covalently bound to the enzyme; Scheme 2). Type I mechanism-based inhibitors, in fact, serve as substrates for the oxidative activity of the enzyme and are converted from 3'-hydroxy to 3'-keto-derivatives. These 3'-keto-derivatives are tightly bound in the NADH form of the enzyme, probably because of the inhibitor-induced conformational change that traps the oxidized inhibitor in the "closed" form of the enzyme.[137]

A striking feature of all these first-generation AdoHcy hydrolase inhibitors is the similarity in their broad-spectrum antiviral activity, indicating a common mechanism of action. These compounds are observed to exhibit more selective activity toward DNA poxviruses (i.e., vaccinia and African swine fever virus), double-stranded ($\pm$) RNA virus (i.e., reoviridae: reo- and rotavirus), and single-stranded ($-$) RNA viruses (i.e., vesicular stomatitis, rabies, parainfluenza and measles).[43,103] Recently, De Clercq's laboratory has extended this antiviral spectrum to include human cytomegalovirus (HCMV), a life-threatening pathogen in immunosuppressed patients.[106] A linear correlation exists between the antiviral activities of these agents and their inhibitory effects on AdoHcy hydrolase.[43,103,106] NpcA is the most potent antiviral agent among the first-generation inhibitors. The IC_{50} values of NpcA for different viruses vary from 0.01 to 0.3 μg/ml, which is about 2000 times lower than that of (S)-DHPA.[102,106] Based on the knowledge that these viruses not only require 5'-capped, methylated structures on their mRNA for translation but also contain virus-specific AdoMet-dependent mRNA methyltransferases, we have hypothesized that the mechanism of antiviral action of AdoHcy hydrolase inhibitors involves an inhibition of the methylation of the capped structure on viral mRNA. As a result, the translation of viral proteins would also be inhibited, thereby suppressing viral replication.
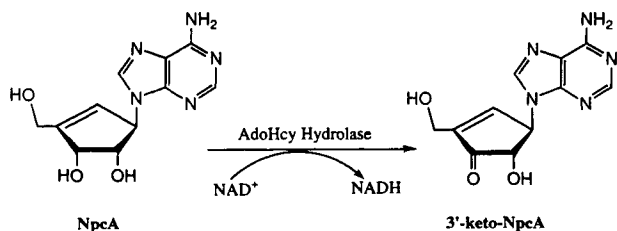
Scheme 2. Mechanism of AdoHcy hydrolase inhibition by Type I mechanism-based inhibitors.

It has been demonstrated that the inhibition of virus multiplication by NpcA coincides with a rapid inhibition of AdoHcy hydrolase activity in the vaccinia virus-infected cells and a subsequent tenfold increase in the intracellular Ado-Hcy/AdoMet ratio.[39] Moreover, it was shown that Ado dialdehyde was most effective when administered within the first 8 hours after infection, implying that early events in viral replication are the site of action.[37] These findings are in agreement with the observation that synthesis of early virus-specific proteins (i.e., based on incorporation of [³⁵S]methionine) is dramatically inhibited by a 12-hour exposure to Ado dialdehyde (to elevate AdoHcy/AdoMet) prior to infection. It is interesting that this treatment was not observed to inhibit cellular protein synthesis, nor did it block the characteristic shut-down in host translation related to the vaccinia infection.[138] In addition, we determined that the incorporation of [³H]methyl groups into RNA isolated from vaccinia virus-infected, Ado dialde-hyde-treated cells was inhibited by approximately 30% in the cytoplasmic fraction and approximately 15% in the poly A⁺-mRNA fraction compared to untreated controls. These data provide additional evidence in support of a mechanism involving inhibition of the methylation of the mRNA capped structure, which results in suppressed translation of viral proteins essential for viral replication.[37,38]

Unfortunately, inhibitors of this generation have a common problem of cellular toxicity, which precludes the clinical use of these compounds as antiviral agents. Is their toxicity due to inhibition of AdoHcy hydrolase or to interactions with other enzymes? Clues concerning the nature of this toxicity were derived from cellular metabolism studies on NpcA (Scheme 3) and Ari. These studies showed that multifunctional metabolic activity is the major cause of toxicity. A concentration as low as 0.1 μM NpcA was shown to inactivate 90% of the AdoHcy hydrolase in mouse L929 fibroblast cells, resulting in a marked increase in cellular Ado-
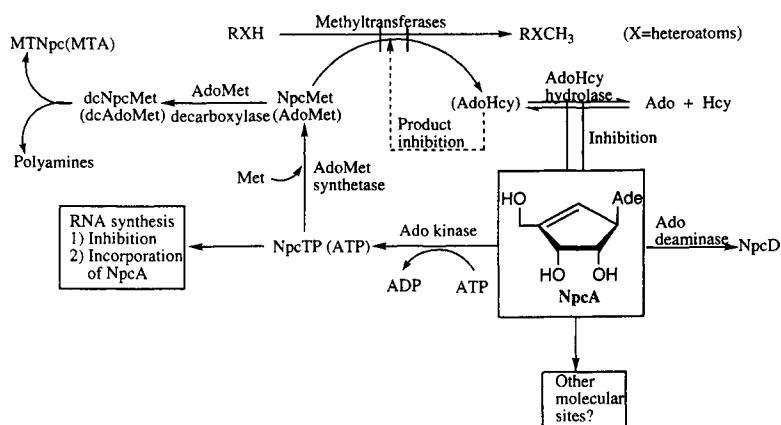
Scheme 3. Metabolic pathways of NpcA (normal Ado metabolites in parentheses).

Hcy/AdoMet levels.[39] Although it is not a substrate for the AdoHcy hydrolase, NpcA was shown to serve as a substrate for Ado deaminase. NpcA is converted by Ado deaminase to the biologically inactive NpcD, the carbocyclic analog of inosine.[36,139] This metabolic route does not seem to be important in pharmacology because coadministration of deoxycoformycin or erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) (both Ado deaminase inhibitors) did not potentiate the effects of NpcA in several different cell lines.[36,140,141] However, it may be important in vivo (e.g., NpcA was not effective in protecting newborn mice against a lethal infection with vesicular stomatitis virus).[102]

NpcA also is a substrate for Ado kinase, leading to the formation of the 5'-triphosphate analog (NpcTP), which is subsequently utilized as a substrate by methionine adenosyltransferase to generate the corresponding AdoMet derivative, S-neplanocylmethionine (NpcMet).[140-144] Metabolism of NpcA by this phosphorylation pathway has been proposed as the mechanism by which this agent produces cytotoxicity.[141] Deleterious effects (e.g., cytotoxicity), which arise because of the formation of these metabolites, are apparently a function of the cell line employed. For instance, cytocidal activity in HT-29 human colon carcinoma cells seems to result from formation of NpcMet.[145] In murine leukemia cells, a NpcA-resistant cell line possessed decreased Ado kinase activity, suggesting that 5'-phosphorylation leads to the antitumor activity observed in the normal cell line.[144] In contrast, Chinese hamster ovary (CHO) cells are known to metabolize NpcA to NpcTP, but an Ado kinase mutant (AdoK⁻) cell line (which produced little, if any, NpcTP) was only slightly more resistant to NpcA treatment.[140] Not much is known about the mechanism of toxicity of NpcTP, except that it is converted to NpcMet and is minimally incorporated into RNA.[141] However, given the ubiquity of the involvement of ATP in biological processes, it would not be surprising to find NpcTP interfering with other fundamental cellular processes.

With respect to the toxicity of NpcMet per se, studies[141,145] suggest that it may inhibit cellular RNA methylation (HT-29 cells) and that NpcA may exert its toxic effect through this metabolite. To date, the effect of NpcMet on mRNA methyltransferases has not been examined. NpcMet is a weak inhibitor of cellular lipid methylation and protein carboxymethylation and is not a substrate for either enzyme involved in these transformations.[36] NpcMet also is neither a substrate nor an inhibitor of AdoMet decarboxylase[36]; however, it did serve as a substrate for catechol-O-methyltransferase (COMT).[142] The assumed formation of NpcHcy in the COMT case raises the question of whether this metabolite plays a role in the observed antiviral activity and/or cytotoxic effects of NpcA in some cell lines by inhibition of viral or cellular methyltransferases.

Ari also exhibits significant cytotoxicity.[38] The metabolism of Ari is somewhat similar to that of NpcA. Kinases also metabolize Ari to its 5'-phosphate derivatives,[146,147] and these nucleotides have been implicated in cellular toxicity.[148,149] However, Ari apparently kills cells by different mechanisms in Ado kinase-deficient (AdoK⁻) and normal (AdoK⁺) cell lines.[150] In AdoK⁺ cells, the phosphate metabo-

lites of Ari are presumably responsible for the toxicity, whereas in the AdoK⁻ cell, AdoHcy hydrolase inhibition may be the cause. Complete inhibition of AdoHcy hydrolase has been implicated in cytotoxicity, and this could be due, at least in part, to Hcy depletion.[151,152] The hydrolysis of AdoHcy is the only source of Hcy in mammalian cells,[153] and Hcy, by conversion to methionine, helps to regenerate the tetrahydrofolate needed for purine and pyrimidine de novo synthesis.[154] Hence, a depletion of Hcy results in an inhibition of nucleic acid biosynthesis. On the other hand, the importance of this Hcy depletion in the cytotoxicity of hydrolase inhibitors can be judged by the observation that, contrary to expectation, cotreatment of these inhibitors with Hcy potentiated their cytotoxic effect (vide infra).[116,155,156]

Another Ari metabolite, carbocylic GMP, is a possible cause of toxicity. The 5′-monophosphate of Ari serves as a substrate for AMP deaminase, converting it to the IMP analog of Ari.[149,157] This metabolite is then transformed to phosphates of carbocyclic guanosine. The carbocyclic analog of GMP is a good inhibitor of hypoxanthine-(guanine) phosphoribosyltransferase,[149] an important enzyme in the purine salvage pathway. This explains the complete blockade of the utilization of hypoxanthine and guanine by cells upon treatment with Ari.[148,149]

Based on the above information that the antiviral action of NpcA is due to an inhibitory effect on AdoHcy hydrolase whereas the cytotoxic action depends on phosphorylation to the corresponding triphosphate, it should be possible to design NpcA analogs that are endowed with antiviral properties while lacking cytotoxicity, thus achieving a better selectivity index (SI) than that of NpcA itself. This hypothesis has led to the design and synthesis of monofunctional analogs of NpcA and Ari, which we call second-generation inhibitors of AdoHcy hydrolase.

B. Second-Generation Inhibitors

In an attempt to design more specific inhibitors of AdoHcy hydrolase with a minimum of other metabolic effects (e.g., Ado deaminase and Ado kinase), NpcA and Ari analogs have been designed using two different approaches. One approach involved replacing the adenine moiety of NpcA and Ari with 3-deazaadenine, resulting in C^3-NpcA.[145,158] and C^3-Ari,[159] respectively (Figure 3). Another approach involved removing the 4′-hydroxymethyl substituent, which would preclude 5′-phosphorylation by Ado kinase and deamination by Ado deaminase.[160] The resulting analogs of NpcA, DHCeA, and its 3-deaza counterpart (C^3-DHCeA), and their saturated counterparts (DHCaA and C^3-DHCaA)[134,161] (Figure 3) were not substrates for either Ado deaminase or Ado kinase, but they retained potent inhibitory activity against AdoHcy hydrolase.[104,147,162–164] Inhibition of AdoHcy hydrolase by C^3-NpcA and C^3-Ari was reported to be reversible and competitive, with K_i values of 0.05 and 3 nM, respectively, when assayed with hamster liver AdoHcy hydrolase.[158,159,165] In contrast, DHCeA, C^3-DHCeA, DHCaA, and C^3-DHCaA have been shown to inactivate AdoHcy hydrolase irreversibly by a type I mechanism.[166] These inhibitors serve as substrates for the enzyme's oxidative
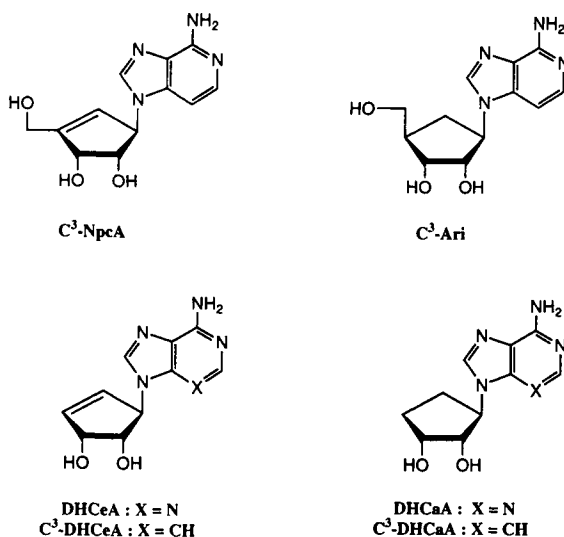
Figure 3. Second-generation AdoHcy hydrolase inhibitors.

activity, resulting in reduction of the enzyme-bound NAD^+ to NADH (inactive). The reaction stops at this point since these inhibitors are not substrates for the "hydrolytic activity" of the enzyme. Direct evidence in support of this mechanism was provided when the inactivation of AdoHcy hydrolase by DHCeA allowed isolation of 3'-keto-DHCeA from the inactivated enzyme after the protein was denatured by treatment with $HClO_4$.[136,166] The inhibitory activity of these compounds was evaluated with recombinant rat liver AdoHcy hydrolase, and the K_i and k_{inact} values were determined to be 62 nM and 0.04 min^{-1} for DHCeA, 85 nM and 0.038 min^{-1} for C^3-DHCeA, 51 nM and 0.25 min^{-1} for DHCaA, and 88 nM and 0.28 min^{-1} for C^3-DHCaA, respectively.[147]

It has been demonstrated that these second-generation AdoHcy hydrolase inhibitors exhibit broad-spectrum antiviral activity, whereas their cytotoxicity is considerably lower than that of the parent compounds (Table 1).[158,159,162] For example, NpcA is a more potent inhibitor of vaccinia virus replication (IC_{50}) in murine L929 fibroblast cells than DHCeA by a factor of 3.5; however, DHCeA is 34 times less cytotoxic (measured as ID_{50} value, the concentration of drug that causes 50% inhibition of cellular replication).[104] DHCeA is, therefore, a better antiviral agent than NpcA by a tenfold increase in the selectivity index (SI = ID_{50}/IC_{50}). By this criterion, the Ari analogs DHCaA and C^3-DHCaA also were much better antiviral agents than Ari by 670- and 330-fold increases in their SI values, respectively.[118]

More recently, antiviral activities of the second-generation inhibitors against African swine fever virus (ASFV) replication in Vero cells were reported.[167] As

Table 1. Comparison of Antiviral Potency, Cytotoxicity, and Selectivity of Second-Generation AdoHcy Hydrolase Inhibitors with Their Parent Compounds Against Vaccinia Virus (VV) Replication in Murine L929 Cells[19,104,118] and African Swine Fever Virus (ASFV) Replication in CCL-81 Vero Cells[167]

Compounds	Antiviral Activity (IC_{50}, μM)^a		ID_{50} (analog) over ID_{50} (parent)		Cytotoxicity (ID_{50}, μM)^b		ID_{50} (analog) over ID_{50} (parent)		Selectivity Index ID_{50}/IC_{50}	
	VV	ASFV	VV	ASFV	L929	CCL-81	VV	ASFV	VV	ASFV
NpcA	0.08	0.57	1	1	0.5	38	1	1	6	66
DHCeA	0.28	2.15	3.5	3.8	17	858.4	34	22.6	61	400
C³-DHCeA	0.95	4.31	11.9	7.6	56	858.4	112	22.6	59	200
Ari	6.62		1		4.3		1		0.64	
DHCaA	0.17	1.29	0.03		73	858.4	17		429	667
C³-DHCaA	0.13	0.34	0.02		27.4	858.4	6		211	2500

Notes: ^{a}Antiviral concentration required to effect a 50% reduction in plaque formation.
^{b}Cytotoxic concentration inhibiting 50% of cell growth.

shown in Table 1, the SI value for NpcA is only 66, whereas DHCeA (SI = 400), C³-DHCeA (SI = 200), DHCaA (SI = 667), and C³-DHCaA (SI = 2500) have much higher SI values and thus are much less cytotoxic. The mechanism of antiviral action of the second-generation inhibitors has been demonstrated to be based on the inhibition of AdoHcy hydrolase, resulting in the elevation of intracellular AdoHcy/AdoMet ratios and subsequent suppression of methylation reactions needed for viral mRNA maturation.[18,38,162] Elimination of Ado kinase and Ado deaminase substrate activities apparently led to the reduced toxicity of these inhibitors.[18]

The elucidation of the mechanism by which NpcA and DHCeA cause inactivation of AdoHcy hydrolase[135,136,166] led several other research groups to synthesize potential type II mechanism-based AdoHcy hydrolase inhibitors. Type II mechanism-based inhibitors of this enzyme are defined as compounds that are catalytically activated and subsequently become covalently bound to the enzyme.[18] Initial attempts to prepare type II mechanism-based inhibitors tried to exploit the oxidative activity of the enzyme to generate an electrophilic site on the inhibitor that could react with a protein nucleophile.[18] For example, (Z)-4',5'-didehydro-5'-deoxy-5'-fluoroadenosine (ZDDFA) was synthesized[168] and shown to be a potent inhibitor of AdoHcy hydrolase.[168–171] Enzyme inhibition was accompanied by reduction of NAD^+ to NADH and release of fluoride ion, suggesting that ZDDFA might function by a type II mechanism (Scheme 4, pathway b and b'). However, it was recently shown that the mechanism of inactivation of AdoHcy hydrolase by ZDDFA involves rapid addition of water at the 5' position of ZDDFA (hydrolytic activity)
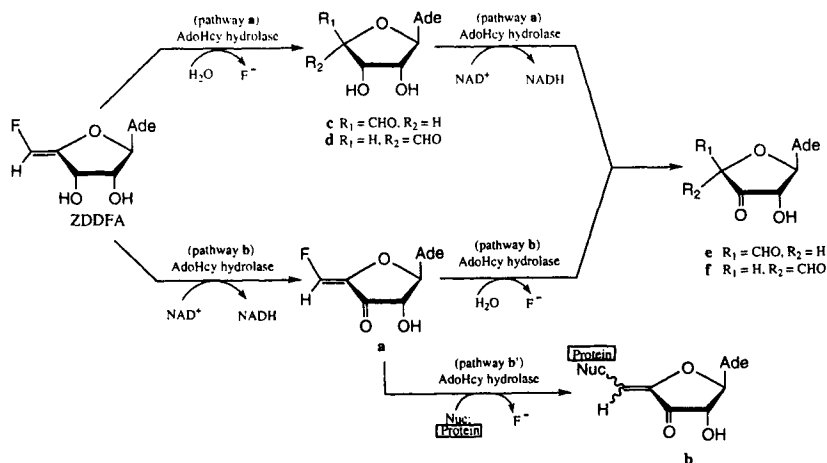
Scheme 4. Possible mechanisms by which ZDDFA inactivates AdoHcy hydrolase.

and elimination of fluoride ion, resulting in the formation of the 5′-carboxaldehydes **c** and **d** (Scheme 4, pathway a).[171] The 5′-carboxaldehydes are oxidized (oxidative activity) in a slower step to 3′-keto-5′-carboxaldehydes **e** and **f** by reduction of the enzyme-bound NAD⁺ to NADH. Intermediate carboxaldehydes **c** and **d** were synthesized independently and proved to be potent type I mechanism-based inhibitors.[172] Carboxaldehyde **c** and ZDDFA have identical K_i values of 40 nM, but the k_{inact} value for carboxaldehyde **c** is 8 times greater than that of ZDDFA.[171] This result shows that ZDDFA is simply a "pro-inhibitor" for a type I mechanism-based inhibitor (Ado 5′-carboxaldehyde) of AdoHcy hydrolase. The unique aspect of this mechanism is that the conversion of the "pro-inhibitor" (ZDDFA) to the "inhibitor" (Ado 5′-carboxaldehyde) actually occurs at the enzyme active site, utilizing the hydrolytic activity of the enzyme. These observations were particularly significant because they showed for the first time that the "hydrolytic activity" of the enzyme can function independently of the "oxidative activity."

C. Third-Generation Inhibitors

Recently, we have synthesized so-called third-generation AdoHcy hydrolase inhibitors, which were designed to use the "hydrolytic activity" of the enzyme to convert a prodrug to a potent drug in the active site of the enzyme. Based on the mechanism elucidated for the ZDDFA-induced inactivation of AdoHcy hydrolase, we initiated attempts to use the hydrolytic activity of this enzyme to catalyze formation of type I mechanism-based inhibitors (e.g., ZDDFA conversion to Ado-5′-carboxaldehyde), a prodrug strategy, and to catalyze formation of strong
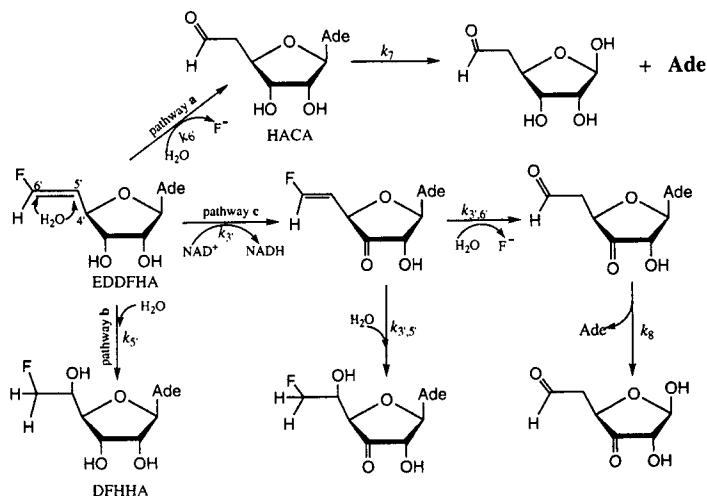
Scheme 5. Mechanism of inactivation of AdoHcy hydrolase by EDDFHA.

electrophiles that could covalently modify the enzyme (type II mechanism-based inhibition), a k_{cat} or suicide inhibition strategy.

The observations with ZDDFA were substantiated recently when it was shown that (E)-5',6'-didehydro-6'-deoxy-6'-halo-homoadenosines (EDDHHAs) also are substrates for the hydrolytic activity of AdoHcy hydrolase.[173,174] Here, the hydrolytic activity is defined as the ability of the enzyme to catalyze addition of water to the 5',6'-bond of EDDHHAs. Scheme 5 shows the mechanism by which the fluorine derivative (EDDFHA) is processed by AdoHcy hydrolase. Incubation of EDDFHA with AdoHcy hydrolase produces a large molar excess of hydrolytic products (e.g., fluoride ion, Ade derived from chemical degradation of homoadenosine 6'-carboxaldehyde (HACA), and 6'-deoxy-6'-fluoro-5'-hydroxyhomoadenosine (DFHHA)) accompanied by a slow irreversible inactivation of the enzyme.[174] The enzyme inactivation was shown to be time-dependent, biphasic, and concomitant with the reduction of enzyme-bound NAD^+ to NADH. The reaction of EDDFHA with AdoHcy hydrolase was shown to proceed by three pathways: pathway a, water attack at the 6' position of EDDFHA and elimination of fluoride ion results in the formation of HACA, which degrades chemically to form Ade; pathway b, water attack at the 5' position of EDDFHA results in the formation of DFHHA; and pathway c, oxidation of EDDFHA results in formation of the NADH form of the enzyme (inactive) and 3'-keto-EDDFHA, which could react with water at either the C5' or C6' positions. The partition ratios among the three pathways were determined to be $k_3:k_{6'}:Ik_{5'} = 1:29:79$, with one lethal event (enzyme inactivation) occurring every 108 nonlethal turnovers. The K_i and k_{inact} ($k_{3'}$) values for EDDFHA

were determined to be 1.3 µM and 0.011 min^{-1}, respectively. The relatively large K_i and small k_{inact} values, which indicate that EDDFHA is a poor inhibitor of AdoHcy hydrolase, could be explained by the efficiencies of pathways a and b, which are nonlethal transformation of EDDFHA. In addition, the reaction products (Ade and DFHHA) from the hydrolytic activity of the enzyme effectively compete with EDDFHA for binding with AdoHcy hydrolase. Similar results were obtained with the Cl, Br, and I derivatives of EDDHHA. The kinetic constants (K_i and k_{inact}) for these EDDHHA derivatives were 96 nM and 0.058 min^{-1} for I, 134 nM and 0.037 min^{-1} for Br, and 110 nM and 0.015 min^{-1} for Cl, respectively.[173] The ratios of k_5/k_6, depend strongly on the properties of the halogen at C6', and were shown to be in the order F > Cl > Br > I.[173,175] The partition ratios (k_5, + k_6,)/k_3, (the ratios of turnover events to inactivation events) are also in the order F > Cl > Br > I. Although the EDDHHAs are not very potent inhibitors of AdoHcy hydrolase, we feel that these observations have suggested alternative ways by which the "hydrolytic activity" could be used to our advantage in designing inhibitors of this enzyme.

The third-generation AdoHcy hydrolase inhibitors shown in Figure 4 have a common feature, which is that they are substrates or products of the hydrolytic activity of the enzyme. They are converted to the active species, Ado 5'-carboxaldehyde (e.g., ZDDFA), or homoadenosine 6'-carboxaldehyde (e.g., EDDHHA) in the active site of the enzyme by catalysis with the enzyme's hydrolytic activity or in aqueous buffer by spontaneous hydrolysis (e.g., 5'-S-(alky and aryl)-5'-fluoro-5'-thioadenosines (AFTA)). In contrast, (Z)-4',5'-didehydro-5'-deoxy-5'-fluoraris-

ZDDFA : X = O, L = F
ZDDFAri : X = CH₂, L = F
L also can be: Cl

EDDFHA : L = F
EDDHHAs : L = F, Cl, Br, I

Ado 5'-carboxaldehyde: X = O
Ari 5'-carboxaldehyde: X = CH₂

DMOA

4',5'-didehydro-5'-methoxyAdo

AFTA
a : R = C₆H₅
b : R = (4)CH₃OC₆H₄
c : R = (4)ClC₆H₄
d : R = CH₃

ACAO

Ado 5'-carboxaldehyde oxime
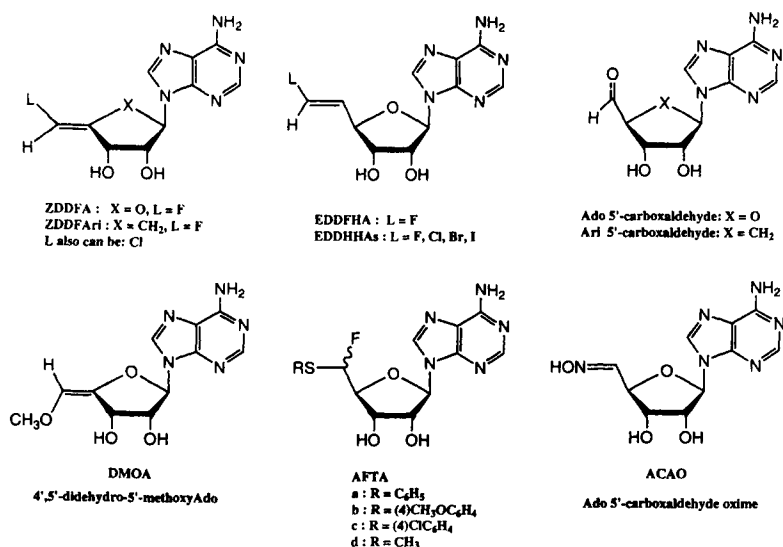
Figure 4. Third-generation AdoHcy hydrolase inhibitors.

teromycin (ZDDFAri) was not a substrate for the hydrolytic activity of the enzyme since incubation of ZDDFAri with AdoHcy hydrolase did not result in the release of fluoride ion.[176] It is possible that enzyme-mediated protonation of the ribosyl ring oxygen of ZDDFA could enhance the electrophilicity of the 5′ carbon; thus, the 5′ position would be more susceptible to attack by water sequestered at the active site of the enzyme. Nevertheless, both ZDDFAri and Ari 5′-carboxaldehyde were shown to be potent inhibitors of AdoHcy hydrolase, with K_i and k_{inact} values of 87 nM and 0.05 min^{-1} for ZDDFAri,[176] and 273 nM and 1.2 min^{-1} for Ari 5′-carboxaldehyde,[177] respectively, when assayed against recombinant rat liver AdoHcy hydrolase.

The AFTAs were unstable in aqueous solutions and spontaneously degraded to Ado 5′-carboxaldehyde via a consecutive reaction mechanism of

$$\text{AFTA} \xrightarrow{k_1} \text{Int} \xrightarrow{k_2} \text{Ado 5′-carboxaldehyde}$$
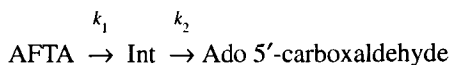
as shown in Scheme 6.[178] The k_1 and k_2 values for AFTA (b) were estimated to be 0.1 min^{-1} and 0.15 min^{-1}, respectively. Rates for solvolysis of the AFTAs were in the order AFTA (d) >> AFTA (b) ≥ AFTA (a) > AFTA (c). All of the AFTAs were shown to produce time- and concentration-dependent inactivation of AdoHcy hydrolase. The apparent K_i value observed for AFTA (b) (K_i = 300 nM) toward bovine liver AdoHcy hydrolase was substantially greater than that observed for AFTA (c) (K_i = 21.4 nM). This might reflect the fact that hydrolysis of AFTA (b) proceeds via formation of an observed intermediate, which is unlikely to be an inhibitor of AdoHcy hydrolase but might be stabilized by interaction with the enzyme. In contrast, degradation of AFTA (c) might result in higher intermediate concentration of the active AdoHcy hydrolase inhibitor Ado 5′-carboxaldehyde. Unlike ZDDFA, which functions as a prodrug of the active 5′-aldehyde-derived type I mechanism-based inhibitors, the AFTAs (which are two-stage synthetic
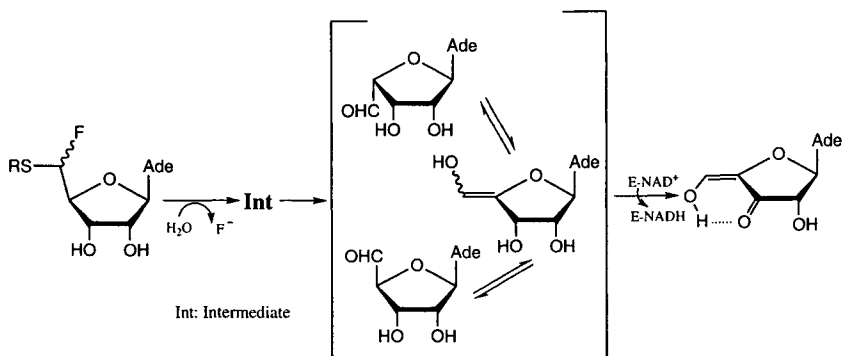
Scheme 6. Mechanism of AdoHcy hydrolase inactivation by AFTA.

precursors, oxidation and thermolysis, of the fluorovinyl analogs) appear to function as "spontaneous solvolysis prodrugs" of the same inhibitor(s).

Interestingly, 4',5'-didehydro-5'-methoxyAdo (DMOA)[178] and Ado 5'-carboxaldehyde oxime (ACAO) also were shown to be potent inhibitors of AdoHcy hydrolase. Inactivation of the enzyme by DMOA and ACAO was shown to follow the ZDDFA-type mechanism for inactivation of AdoHcy hydrolase,[178] i.e., DMOA and ACAO were first converted to the active species by the enzyme's hydrolytic activity. The resulting Ado 5'-carboxaldehyde then functions as a type I mechanism-based inhibitor of the enzyme. The K_i and k_{inact} values for DMOA and ACAO were determined to be 1.59 μM and 0.13 min^{-1}, and 0.22 μM and 0.13 min^{-1}, respectively, toward recombinant human placental AdoHcy hydrolase.

Antiviral activity data for the third-generation AdoHcy hydrolase inhibitors are limited at present. ZDDFA was a potent antiviral agent against vaccinia virus.[19] The IC_{50} and ID_{50} values were determined to be 0.05 and 15.6 μM, respectively, with a SI value of 312. ZDDFA has potential as an antiretroviral agent against Moloney murine leukemia virus (MLV).[179] The IC_{50} value of ZDDFA against MLV replication in murine fibroblasts (SC-1) cells was 0.18 μM, and the ID_{50} for SC-1 cells growth was 214 μM, which gives a SI value of 1189. The antiretroviral activity of ZDDFA has been demonstrated to correlate with its ability to inhibit AdoHcy hydrolase and maintain elevated intracellular AdoHcy levels.[179]

ZDDFA also was an effective antiviral agent against African swine fever virus (ASFV).[167] The mechanism of the anti-ASFV action of ZDDFA is postulated to depend on the inhibition of AdoHcy hydrolase, which results in suppression of viral mRNA maturation. The IC_{50} value of ZDDFA against ASFV replication in Vero cells was estimated to be 0.077 μM, and the ID_{50} value was 52.1 μM, which gives a SI value of 677.

Comprehensive evaluation of the present third-generation AdoHcy hydrolase inhibitors as potential antiviral agents is under way. The exciting potential for Type II mechanism-based inhibitors that are designed to utilize the hydrolytic activity of AdoHcy hydrolase must await further investigation.

IV. RECENT RELEVANT NUCLEOSIDE CHEMISTRY

Extensive modifications on both the heterocyclic base and ribose moieties have been made in attempts to prepare adenine-type nucleosides that are potent inhibitors of AdoHcy hydrolase and function as antitumor and antiviral agents. This discussion of nucleoside chemistry comprises syntheses of both "carbocyclic sugar" and glycosyl analogs. Earlier work on the synthesis of chiral carbocyclic nucleosides has been reviewed.[180] The present discussion of that area is limited to methodologies recently developed for the synthesis of carbocyclic nucleosides targeted to inhibit AdoHcy hydrolase.

A. Preparation of Carbocyclic Nucleosides from Optically Active Dihydroxycyclopentenones Derived from Carbohydrates

Since the carbocyclic antibiotic NpcA (**25**, Scheme 11) is a potent inhibitor of AdoHcy hydrolase, analogs of **25** lacking the 4'-hydroxymethyl group were designed as potentially less cytotoxic inhibitors that would not be substrates for adenosine kinase and, therefore, would not enter nucleotide analog metabolic pathways. The protected (–)-dihydroxycyclopentenone enantiomer **4** (Scheme 7) and its isopropylidene analog **9** (Scheme 9) were selected as key intermediates.

Stereoselective syntheses of optically pure hydroxylated cyclopentenones have been reported,[181–186] whereas others require enantiomeric resolution.[187–189] A new synthesis of (–)-dihydroxycyclopentenone **4** from D-ribonolactone **1** was developed[190] (Scheme 7). Lactone **1** was converted to the L-erythruronolactone **2** by a known procedure.[191] Treatment of **2** in 2-propanol at reflux with catalytic pyridinium *p*-toluenesulfonate (PTS) gave the L-glycoside **3**, which was treated with lithium dimethyl methylphosphonate to give **4** (65% overall). A plausible mechanism for the conversion of **3** to **4** is shown in Scheme 8. Attack of lithium dimethyl methylphosphonate on the carbonyl group of **3** would result in opening of the lactone ring and elimination of alkoxide to give acyclic intermediate **5**. Base-promoted cyclization of **5** would form cyclopentenone **4**. The enantiomeric (+)-dihydroxycyclopentenone was synthesized from D-mannose by a similar procedure via the D-erythruronolactone[192] (44% overall).[190]

An efficient three-step synthesis of the isopropylidene-protected (–)-dihydroxycyclopentenone **9** from D-lyxose (**6**, Scheme 9) was devised.[193] The methyl 2,3-*O*-isopropylidene-D-lyxofuranoside **7** was oxidized with pyridinium chlorochromate (PCC) to produce lactone **8**. This represents a novel case of oxidative cleavage of a carbon-carbon bond by PCC. Treatment of **8** with lithium dimethyl methylphosphonate gave the desired enone **9** (41% overall). The enantiomer of **9** was prepared from D-ribose in a similar manner.

Stereoselective reduction of **4** with sodium borohydride in the presence of cerium(III) chloride[194] gave cyclopentenol **10** (Scheme 10), which was tosylated to give **11**.[193] The tosylate group was displaced from **11** by adenine or 3-deazaAde, and the products were deprotected with dilute hydrochloric acid to give 9-[(1'*R*, 2'*S*, 3'*R*)-2',3'-dihydroxycyclopent-4'-en-1'-yl]Ade (DHCeA, **12**) and
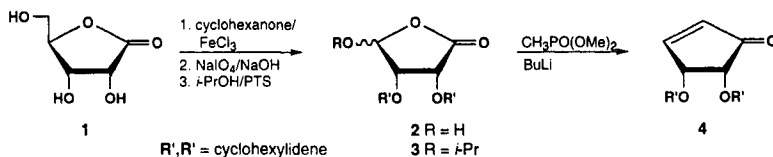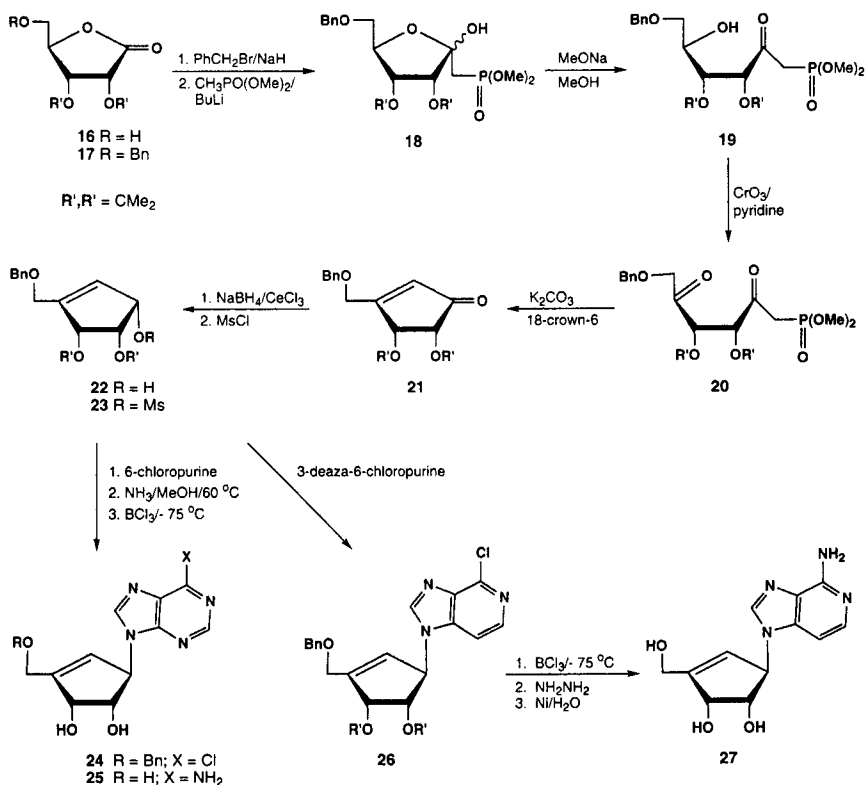
Scheme 7.
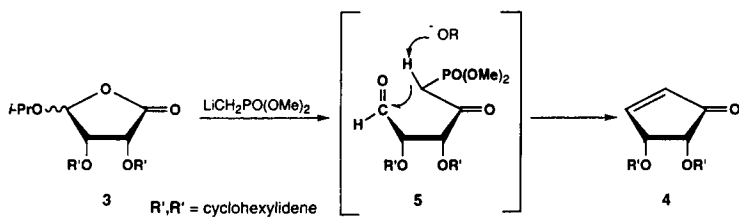
Scheme 8.
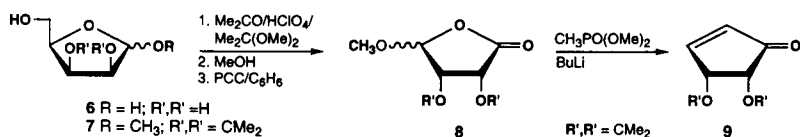
Scheme 9.

3-deazaDHCeA (**13**), respectively. Hydrogenation of **12** and **13** with Adam's catalyst gave 9-[(1′R, 2′S, 3′R)-2′,3′-dihydroxycyclopentan-1′-yl]Ade (DHCaA, **14**) and 3-deazaDHCaA (**15**), respectively. A dihydroxycyclopentenone synthon also was used to prepare the queuine base of nucleoside Q.[195] Applications of these protected chiral dihydroxycyclopentenones for the synthesis of other natural products should be promising.[182,196]
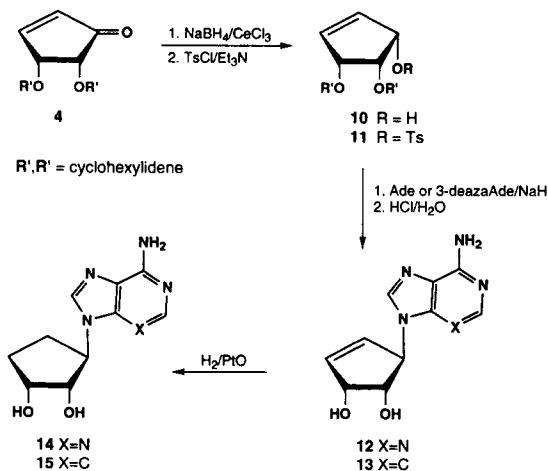
Scheme 10.

B. Efficient Enantiospecific Syntheses of Aristeromycin, Neplanocin A, and Selected 4' Analogs

Ari (**31**) and NpcA (**25**), carbocyclic analogs of Ado, were isolated from *Streptomyces citricolor*[197,198] and *Ampullariella regularis*,[199,200] respectively, and have antitumor and antiviral activity.[43] The first enantiospecific syntheses of **25** and **31** were reported in 1983.[201] Other enantioselective and -specific syntheses of **25**[186,201,202] and **31**[201–205] as well as routes to racemic **31**[205–209] have been developed. Linear and convergent approaches to carbocyclic nucleosides have been reviewed.[180,210] Convergent approaches that give greater flexibility for the synthesis of functionalized carbocyclic moieties have been pursued in searches for more selective chemotherapeutic agents based on the inhibition of AdoHcy hydrolase.

The first convergent synthesis of a carbocyclic nucleoside ((–)-NpcA (**25**)) began[211] with 2,3-*O*-isopropylidene-D-ribono-1,4-lactone (**16**) (Scheme 11). Ben-

Scheme 11.

zylation of **16** and treatment of the 5-*O*-benzyl intermediate **17** with lithium dimethyl methylphosphonate gave the hemiacetal **18**, which was treated with sodium methoxide (to give **19**) and then oxidized with chromium trioxide in pyridine to give diketophosphonate **20**. Intramolecular Wittig-Horner cyclization of **20** afforded cyclopentenone **21**, which was reduced stereoselectively to the α-alcohol **22** with sodium borohydride in the presence of cerium(III) chloride. Mesylation of **22** and S_N2 displacement of mesylate from **23** with 6-chloropurine gave the carbocyclic nucleoside derivative **24**. Treatment of **24** with ammonia and deprotection with boron trichloride gave NpcA (**25**, 1.5% overall from D-ribono-lactone). C^3-NpcA analog[158] **27** was prepared from **23** with the sodium salt of 3-deaza-6-chloropurine via intermediate **26**. The stereochemistry of **27** was confirmed by X-ray crystallography. Analogous approaches have been reviewed.[180] Recently, cyclopentenone **21** was used for the synthesis of "psico-cyclopentenyl" analogs of **25** with a hydroxymethyl substituent at C1'.[212]

A recent total synthesis of NpcA (**25**) and Ari[213,214] (**31**, Scheme 12) has the advantages of fewer steps and greater versatility for the preparation of analogs of both **25** and **31**. This synthesis also employed the key cyclopentenone **4**. Thus, **4** was treated with the hydroxymethyl equivalent lithium bis(*tert*-butoxymethyl)cu-
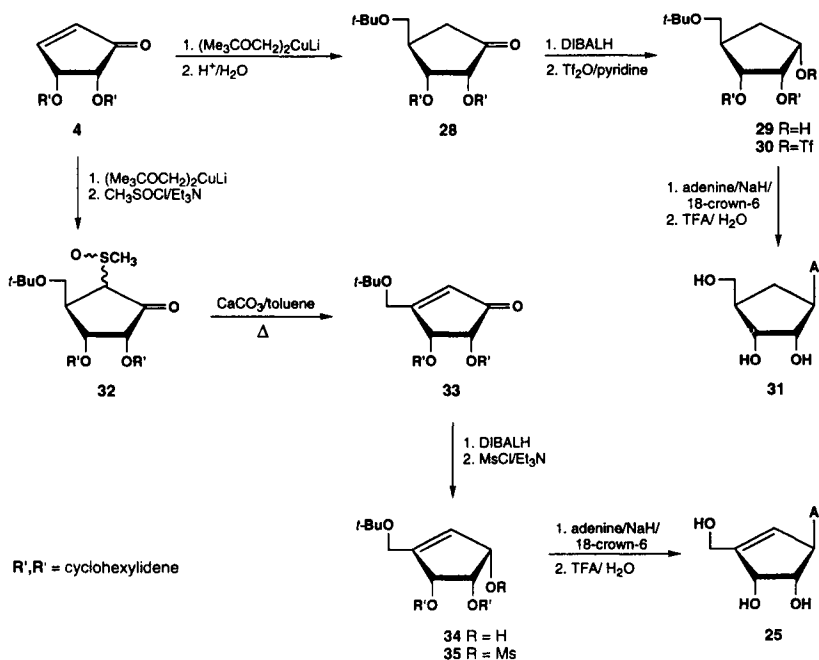
Scheme 12.

prate,[215] and the reaction was quenched with a proton source (e.g., acetic acid) to give the Ari precursor 28, which was diastereomerically pure (^{1}H and ^{13}C NMR). Proton decoupling experiments and molecular modeling indicated that H4 was *trans* to H3 (4R configuration). Unambiguous proof of the structure of 28 was provided by conversion of this ketone to Ari. Reduction of 28 with diisobutylaluminum hydride (DIBALH) gave the α isomer 29 in 92% diastereomeric excess (^{1}H and ^{13}C NMR). The α and β isomers were separated chromatographically and the stereochemistry of each was determined (NMR). A *cis* relationship was indicated for H1 and H2 in the major isomer, confirming that DIBALH had approached selectively from the less hindered face. Alcohol 29 was converted to triflate 30, which was treated with Ade anion, and the product was deprotected to give Ari (31).[214]

Quenching of the above enolate (resulting from conjugate organometallic addition to 4) with methanesulfinyl chloride gave the β-keto sulfoxide 32. Pyrolytic *syn* elimination of methyl sulfenic acid from 32 gave the NpcA precursor cyclopentenone 33. Analogous reduction of 33 gave the α diastereoisomer 34 only. Alcohol 34 was converted to mesylate 35 and treated with adenine anion followed by deprotection to give NpcA (25).[214,216]

This synthetic route is efficient and enantiospecific. It is also divergent and was adapted for the preparation of 4'-modified analogs.[134] Treatment of 4 with lithium dimethylcuprate followed by acidic quenching gave the conjugate addition product 36 (Scheme 13). The use of other organocuprate reagents gave 37 and 38. These 4-substituted cyclopentanones were converted into the Ari analogs 39–41 by the standard synthetic sequence. Treatment of 4 with lithium dimethyl- or diethylcuprate, quenching with methanesulfinyl chloride, and pyrolytic elimination gave the
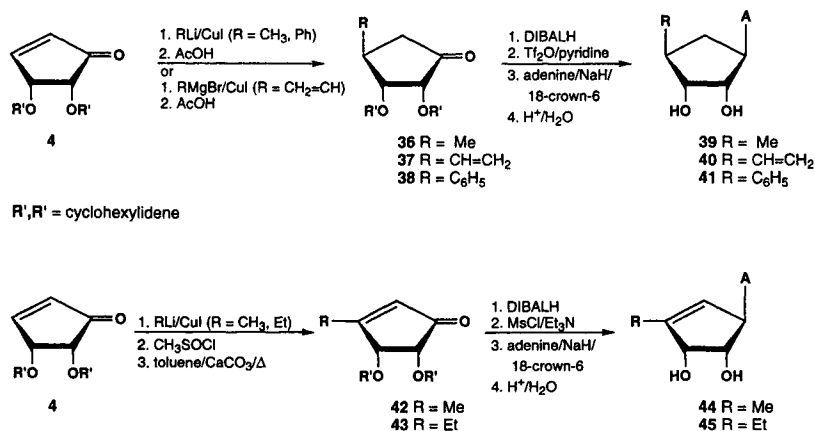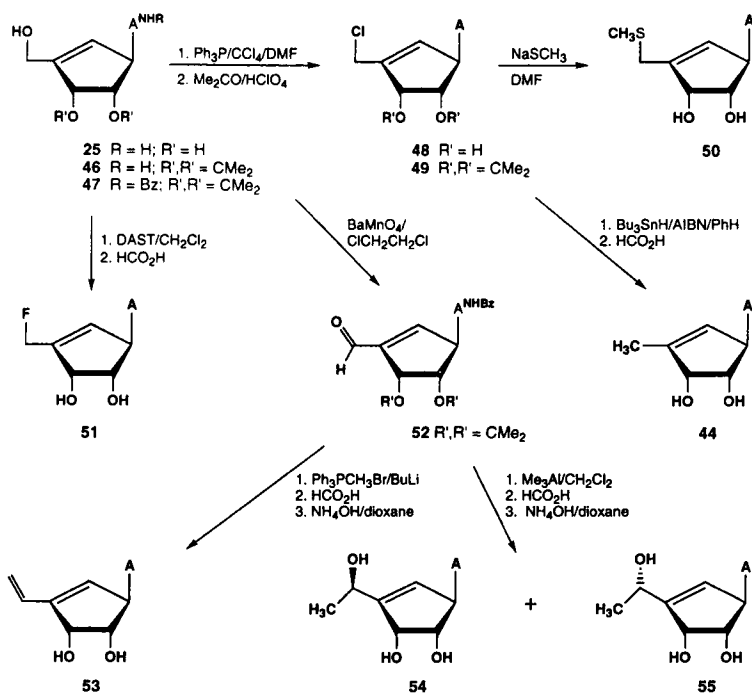

Scheme 13.

respective 4-methyl and -ethyl cyclopentenones **42** and **43**, which were converted into the NpcA analogs **44** and **45**.
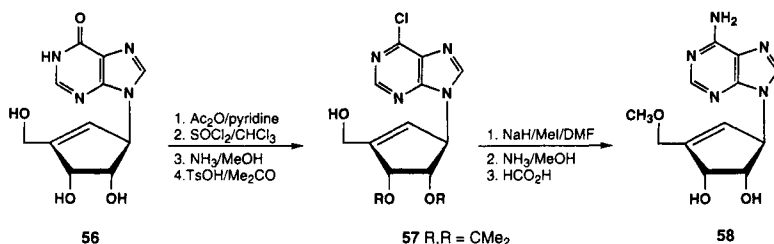
C. Other Aristeromycin and Neplanocin A Analogs

Ari (**31**) and NpcA (**25**) have been used as starting materials for the preparation of analogs in the search for new antiviral agents. Development of the methodology described in Scheme 12 provides synthetic access to **31** and **25** for syntheses of modified derivatives.[180]

Syntheses of 4′,5′-didehydro-5′-deoxy-5′-fluoroAris (**77**, Scheme 18) were reported recently,[176,217] and the preparation of Ari analogs with a hydroxyl group in place of the hydroxymethyl moiety at C4′ was also described.[218] Several 5′-modified derivatives of NpcA (**25**) were prepared from **25**[219] (Scheme 14). Treatment of **25** with triphenylphosphine/tetrachloromethane/DMF gave the 5′-chloro-5′-deoxy derivative **48**. Nucleophilic displacement of chloride from **48** with sodium thiomethoxide gave 5′-S-methyl-5′-thioNpcA (**50**). Treatment of 2′,3′-O-isopropylideneNpcA (**46**) with (diethylamino)sulfur trifluoride (DAST) followed by deprotection gave 5′-deoxy-5′-fluoroNpcA (**51**). The 5′-chloro 2′,3′-O-iso-

25 R = H; R' = H
46 R = H; R',R' = CMe₂
47 R = Bz; R',R' = CMe₂

48 R' = H
49 R',R' = CMe₂

50

51

**52** R',R' = CMe₂

44

53

54

55

Scheme 14.

Scheme 15.

propylidine derivative **49** was subjected to dehalogenation with tributylstannane and deprotected to give 5'-deoxyNpcA (**44**). Oxidation of 6-*N*-benzoyl-2',3'-*O*-isopropylideneNpcA (**47**) with barium permanganate gave the enal **52**. Treatment of this conjugated aldehyde with methylenetriphenylphosphorane and deprotection gave the homovinyl diene **53**. Treatment of **52** with trimethylaluminum gave the 5'-*C*-methyl diastereomers, which were deprotected and resolved by HPLC to give the 5'*R* (**54**) and 5'*S* (**55**) homologues. The 5'-*O*-methylNpcA (**58**, Scheme 15) was prepared by methylation of the 6-chloro 2',3'-*O*-isopropylidene intermediate **57** derived from hypoxanthine analogue **56** (NpcD). Direct methylation of the 5'-hydroxyl group of NpcA with methyl iodide resulted in formation of base alkylation by-products. All of these NpcA congeners showed inhibitory activity against AdoHcy hydrolase.[219]

Two 9-(dihydroxycyclohexenyl and dihydroxycyclohexanyl) derivatives of adenine were prepared as ring-expanded analogs of NpcA and Ari.[220] These syntheses provided potential antiviral agents and afforded a new methodology for the preparation of hydroxylated cyclohexenyl and cyclohexanyl nucleosides. Syntheses of the racemic 9-[(1'*R*,2'*S*,3'*R*)-2',3'-dihydroxycyclohex-4'-en-1'-yl]adenine (**65**) and 9-[(1'*R*,2'*S*,3*R*)-2',3'-dihydroxycyclohexan-1'-yl]adenine (**66**) homologs of DHCeA (**12**) and DHCaA (**14**), respectively, are shown in Scheme 16. Epoxidation of 1,3-cyclohexadiene (**59**) with 3-chloroperoxybenzoic acid (MCPBA) gave (+)-3,4-epoxycyclohexene (**60**). Nucleophilic addition of adenine to the allylic epoxide **60** in the presence of a Pd(0) catalyst gave the *cis* 1,4-alkylated product (+)-**61**. Pd(0) catalysis was essential for the *cis* addition stereochemistry. Treatment of **61** with osmium tetroxide and *N*-methylmorpholine *N*-oxide (NMO) in aqueous acetone gave **62** (95%) by attack of the hydroxylation complex from the less hindered face. The *trans* relationship between the 2',3'-diol and the 4'-hydroxyl group was confirmed by an ¹H NMR NOE experiment with the 2',3'-*O*-isopropylidene compound **63**. The 6-amino group of **63** was protected as its dimethylaminoacetamidine derivative, and the 4'-hydroxyl group was eliminated with DAST in methylene chloride to give **64** (67% for three steps). Deprotection gave **65**, which was hydrogenated to give **66**.
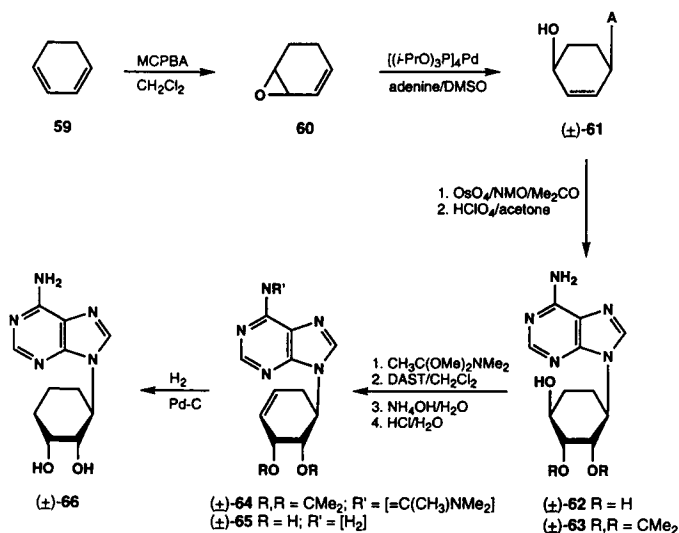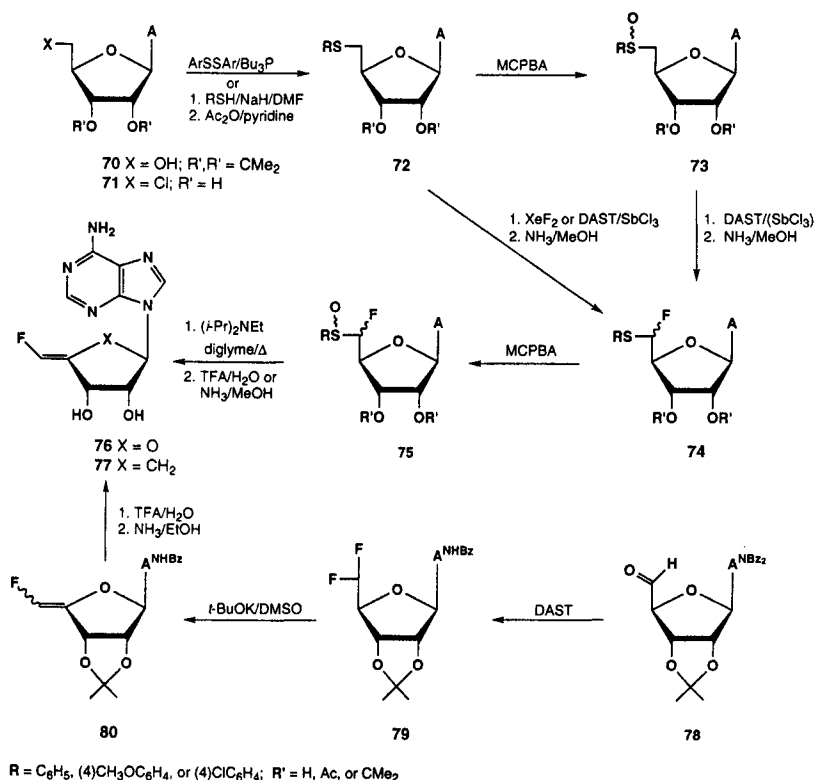
Scheme 16.

D. Syntheses of 5'-S-(Alkyl and Aryl)-5'-fluoro-5'-thioadenosines and Transformations to 5'-Fluoro-4',5'-Unsaturated Nucleosides (Including Carbocyclic Analogs)

Conversion of sulfoxides **68** into α-fluoro thioethers **69** (Scheme 17) with DAST was reported in 1985.[221] This reaction allowed new transformations of thionucleosides into fluoro-substituted nucleosides. Antimony(III) chloride was found to be an effective catalyst for this process,[222,223] and recently it was shown[224] that thioethers **67** were converted directly into α-fluoro thioethers **69** with DAST/SbCl₃.

Scheme 17.

70 X = OH; R',R' = CMe$_2$
71 X = Cl; R' = H

72

73

ArSSAr/Bu$_3$P
or
1. RSH/NaH/DMF
2. Ac$_2$O/pyridine

MCPBA

1. XeF$_2$ or DAST/SbCl$_3$
2. NH$_3$/MeOH

1. DAST/(SbCl$_3$)
2. NH$_3$/MeOH

1. (*i*-Pr)$_2$NEt
diglyme/Δ
2. TFA/H$_2$O or
NH$_3$/MeOH

76 X = O
77 X = CH$_2$

75

MCPBA

74

1. TFA/H$_2$O
2. NH$_3$/EtOH

80

t-BuOK/DMSO

79

DAST

78

R = C$_6$H$_5$, (4)CH$_3$OC$_6$H$_4$, or (4)ClC$_6$H$_4$; R' = H, Ac, or CMe$_2$
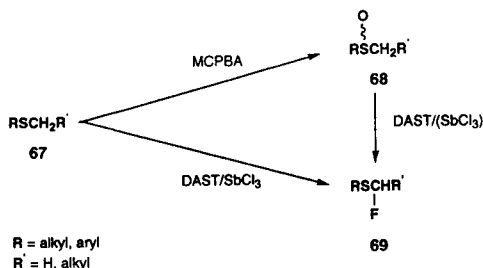
Scheme 18.

This improvement eliminated the need for oxidation of **67** to **68** in the overall transformation of thioethers **67** to α-fluoro thioethers **69**.[224]

The protected 5'-*S*-alkyl(and aryl)-5'-thioAdo starting materials[225] **72** (Scheme 18) for the synthesis of fluoro nucleosides were obtained from Ado by a sequence (~90%) involving 5'-chloro-5'-deoxyadenosine (**71**)[226] or from 2',3'-*O*-isopropylideneadenosine (**70**) by a disulfide/phosphine procedure.[227] The isopropylidene route was employed without isolation of intermediate 5'-α-fluoro thioethers **74** to give the *Z* vinyl fluoride **76** and its *E* isomer (2.5:1) in 22% overall yield.[168,170] Thus, oxidation of **72** (R = 4-methoxyphenyl, R',R'= CMe$_2$) with MCPBA and treatment of the resulting sulfoxide **73** with DAST gave the diastereomeric fluoro thioethers **74**. Oxidation of this mixture to the 5'-α-fluoro sulfoxides **75**, thermal *syn*-elimination of sulfenic acid, deprotection with aqueous acid, and chromatography afforded the *Z* vinyl fluoride **76** and its *E* isomer. Similar chemistry was employed for the synthesis of fluoromethylene analogs of **76**, beginning with 9-(β-D-arabinofuranosyl)Ade and 2'-deoxyAdo.[170] In an alternative

route, the protected Ado 5′-carboxaldehyde **78** was treated with DAST to give difluoride **79** (18%). Dehydrofluorination of **79** with potassium *tert*-butoxide in dimethyl sulfoxide gave the vinyl fluorides **80** (*Z/E*, ~2:1), which were deprotected to give **76** and its *E* isomer.[170]

Efficient syntheses of 5′-*S*-aryl-5′-fluoro-5′-thioAdo analogs **74** were achieved with 2′,3′-di-*O*-acetyl protection, and yields in the crucial fluorination step were improved with the DAST/SbCl₃ combination.[178,228,229] Thus, treatment of sulfoxide **73** (R = 4-methoxyphenyl, R′ = Ac) with DAST/SbCl₃ gave the 5′-fluoro diastereomers **74** (5′*R/S*, ~2:3) in >70% yields. Deacetylation (NH₃/MeOH) of these acid-sensitive derivatives **74** (R′ = Ac) allowed isolation and biological evaluation (vide supra) of the Ado 5′-α-fluoro thioether analogs **74** (R′ = H).[178]

It is noteworthy that the SbCl₃-catalyzed fluorination process is almost as efficient with the 5′-*S*-phenyl sulfoxides **73** (R = phenyl) as with the much more expensive 5′-*S*-(4-methoxyphenyl) analogs. Even the deactivated 4-chlorophenyl sulfoxide **73** (R = 4-chlorophenyl) gave the fluoro diastereomers **74** in good yields with SbCl₃ catalysis, but minor amounts of the deoxygenated thioether precursor **72** were also formed. Deacetylation and fractional crystallization afforded single 5′-fluoro diastereomers **74** in some cases (e.g., R = 4-methoxyphenyl, R′ = H).[178] ¹⁹F NMR peaks for the 5′(*R*)-fluoro diastereomers appeared at lower fields than those of their *S* counterparts, and the absolute configurations were confirmed by X-ray crystallography.[178,230] Similar chemistry was applied for fluorinations at C5′[230] and C2′[231] of uridine thioethers and for preparations of 3′-(fluoromethyl)thio compounds derived from thymidine.[232] Interestingly, fluorinations appeared to proceed exclusively at C2′ with the 2′-*S*-methyl-2′-thiouridine derivatives,[231] whereas the methyl group was the site of fluorination with the 3′-*S*-methyl-3′-thiothymidine analog.[232]

Direct fluorination of 5′-*S*-(4-methoxyphenyl)-5′-thio nucleoside **72** with xenon difluoride proceeded smoothly to give an inverted ratio of fluoro diastereomer **74** (5′*R/S*, ~3:2) with yields comparable to those of the DAST/sulfoxide procedure.[178] It is noteworthy that the direct fluorination of thioethers[224] **72** with DAST/SbCl₃ gave 5′-fluoro diastereomers **74** in good yields and with ratios (5′*R/S*, ~2:3) similar to those found with the DAST/sulfoxide procedure and different than those of the XeF₂/sulfide method. The carbocyclic 4′,5′-didehydro-5′-deoxy-5′-fluoro compound **77** was prepared analogously from aristeromycin.[176,217] Treatment of the (phenyl) 5′-sulfoxide of 2′,3′-*O*-isopropylidenearisteromycin with DAST/SbCl₃ effected fluorination at C5′.

Treatment of 2′,3′-di-*O*-acetyl-5′-*S*-methyl-5′-thioadenosine (**81**, Scheme 19) with XeF₂178 or DAST/SbCl₃224 or its sulfoxide **82** with DAST/SbCl₃178,228 or DAST[233,234] gave regio- and diastereomeric mixtures of 5′-*S*-(fluoromethyl)-5′-thioAdo (**84a**) and 5′-fluoro-5′-*S*-methyl-5′-thioAdo (**85a**) in ~60% combined yield after chromatography. The ratios of the 5′-diastereoisomers, as well as regioisomers, of the fluoro products once again were parallel for the sulfide/DAST[224] and sulfoxide/DAST[178,228,229] reactions (e.g., **85a** (5′*R/S*, ~1:2);
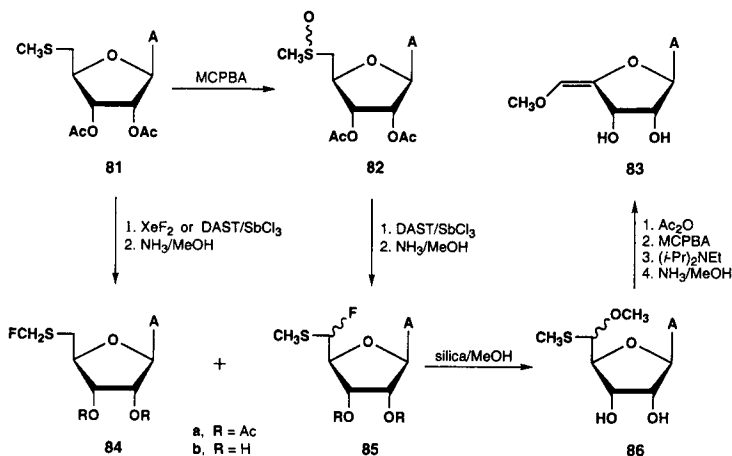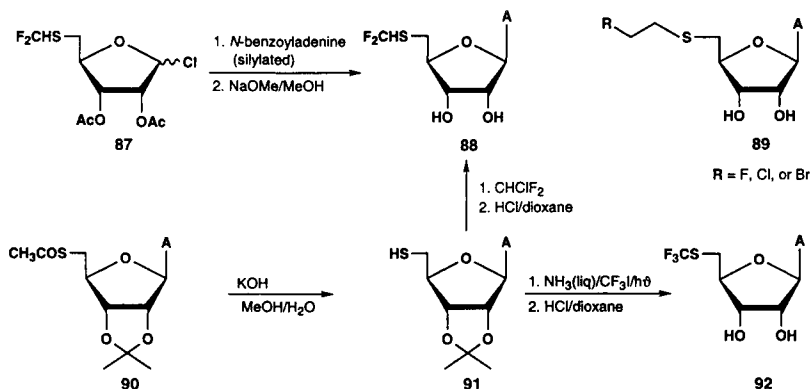
Scheme 19.

85a/84a, ~3:2) and inverted for the sulfide/XeF$_2$178 reaction (**85a** (5'*R/S*, ~1:1); **85a/84a**, ~2:3). Recently it was noted that fluorination of **81** with XeF$_2$ at –60°C gave **84a** selectively.[235] Fluorination of **81** with DAST/SbCl$_3$[224] proceeded quantitatively (^{19}F, ^{1}H NMR), and the ratio of fluoro isomers in the crude reaction mixture was **85a** (5'*R/S*, ~1:1.2); **85a/84a**, ~3.5:1. This procedure was utilized for the synthesis of nucleoside 5'-aldehyde derivatives (vide infra) and the 5'-*O*-methyl enol ether **83**.

The sensitive fluoromethyl thioether **84a** was sufficiently stable for chromatographic purification, deprotection to **84b**, and gentle manipulation. The regioisomeric 5'-fluoro-5'-methylthio diastereomers **85a** were deprotected and then directly converted to mixed methoxy/methylthio acetals **86** on silica gel columns with methanol-containing solvents.[178,228] The deprotected isomers **85b** were isolated by anion exchange chromatography.[224,233] Compounds **84b** and **85b** were also found to be inhibitors of methylthioadenosine phosphorylase and to have antiproliferative properties.[233]

The mixed acetal **86** (~4:1 mixture of diastereomers) was obtained in ~40% yield from **81** by rapid deacetylation of crude **84a/85a** and slow passage of the mixture through a column of silica gel with methanol/chloroform. The **86** mixture was acetylated, oxidized to sulfoxides, thermolyzed, and deacetylated to give the methoxy vinyl ether **83** in low yield.[178] This 5'-*O*-methyl derivative **83** of the hydroxy enol tautomer (**108**, Scheme 22) of adenosine 5'-aldehyde was a time-dependent inactivator of AdoHcy hydrolase.[178] Other methods for the synthesis of 5'-*S*-fluoroalkyl analogs of methylthioAdo (e.g., **88**, **89**, and **92**, Scheme 20) include: (i) coupling of derivatives of 5-deoxy-5-*S*-(mono or difluoro)methyl-5-
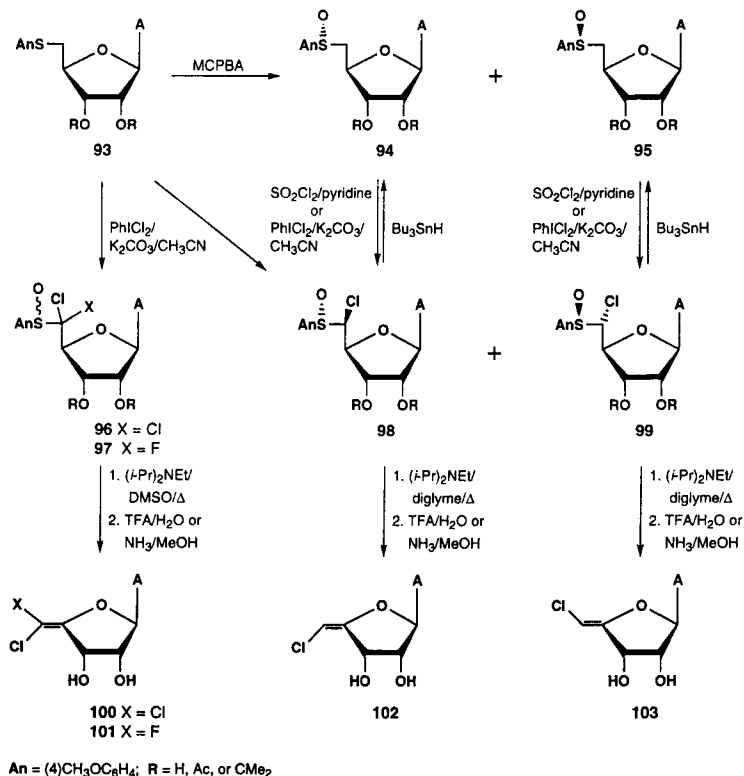
Scheme 20.

thioribose (e.g., **87**) with adenine,[236] (ii) generation of the 5'-thio function of **91** from its 5'-thioacetate **90** under basic conditions and alkylation with fluoroalkyl reagents,[237] (iii) treatment of 5'-deoxy-5'-iodouridine with a mercury(II) tri-fluoromethylthio complex to give the uracil analog of **92**,[238] and (iv) treatment of 5'-S-[(2-hydroxyethyl)]-5'-thioAdo with thionyl chloride, bromide, or DAST to give the corresponding 5'-[(2-haloethyl)thio] derivatives **89**.[239] The chemistry of these and other 5'-modified thionucleosides has been reviewed.[225]

E. Stereochemically Defined Synthesis of 5'-Chloro-4',5'-Unsaturated Adenosines

Treatment of the 2',3'-O-isopropylideneadenosine 5'-sulfoxide diastereomers **94/95** (R,R = CMe$_2$, Scheme 21) with sulfuryl chloride/pyridine[240] gave the α-chlorinated derivatives **98/99**.[170] Thermolysis of the **98/99** mixture, deprotection, and isolation of the major product gave a 5'-chloromethylene analog, **102**, whose stereochemistry was erroneously assigned as 5'(Z).[170] Treatment of the 2',3'-di-O-acetyl thioether **93** or its diastereomeric sulfoxides **94/95** (R = Ac) with iodoben-zene dichloride[241] resulted in formation of the 5'-chloro (and 5',5'-dichloro)-5'-deoxy-5'-[(4-methoxyphenyl)sulfinyl]Ados (**96/98/99**, R = Ac).[242]

Sulfoxide diastereomers **94**(S$_R$) and **95**(S$_S$) (R = Ac) underwent α-chlorination with iodobenzene dichloride with predominant retention of configuration at sulfur to give **98**(5'S, S$_S$) and **99**(5'R, S$_R$) (R = Ac), respectively, as major products. Configurations at sulfur and C5' were determined by X-ray crystallography and radical-mediated reductive dechlorination correlations.[242] Thermolysis and deace-tylation gave the 5'-chloromethylene **102**(E) and **103**(Z) and 5'-dichloromethylene **100** derivatives.[242] It is noteworthy that attempts (e.g., reaction of **75** with DAST/SbCl$_3$ or **74** with XeF$_2$) to synthesize nucleoside α,α-difluoro 5'-thioethers

An = (4)CH₃OC₆H₄; R = H, Ac, or CMe₂

Scheme 21.

failed.[178,230] However, chlorination of a protected α-fluoro sulfoxide **75** (R′,R′ = CMe₂) with sulfuryl chloride gave the dihalogenated (α-chloro-α-fluoro) sulfoxide **97** (R,R = CMe₂).[170] Thermal elimination of sulfenic acid from **97** and deprotection gave the *E*-isomer **101**. Photoisomerization of **101** afforded the *Z* compound.[170] The 5′-chloromethylene compound **103**(*Z*) is a potent time-dependent inactivator of AdoHcy hydrolase.[229,242] It was demonstrated recently that the *Z*-fluoro analogue **76** is a prodrug of the potent AdoHcy hydrolase inhibitory "Ado 5′-carboxalde-hyde," which are generated (vide supra) by the "hydrolytic activity" of that enzyme.[171]

F. Synthesis of "Adenosine 5′-carboxaldehydes" and Their Oximes

Oxidation of protected Ado derivatives to give "Ado 5′-aldehyde" intermediates has been used frequently in synthetic nucleoside chemistry.[243] However, such aldehydes form hydrates and undergo side-reactions and isomerizations readily,
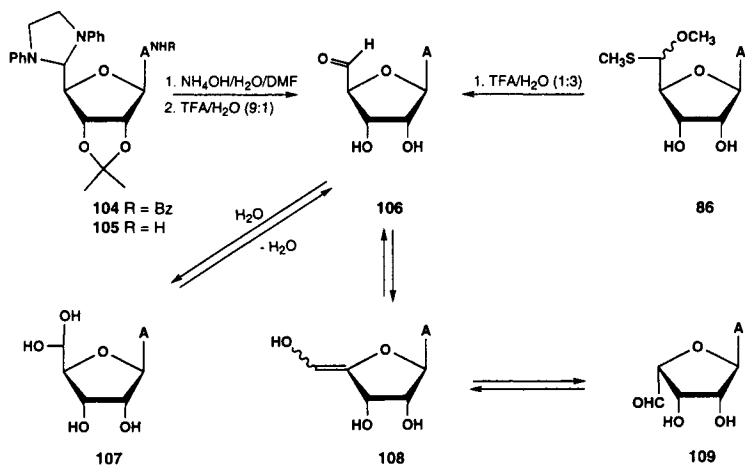
Scheme 22.

especially when deprotected. Since it was discovered that nucleoside vinyl fluoride **76** liberated fluoride anion upon incubation with AdoHcy hydrolase,[171] the mode of time-dependent inactivation of the enzyme became of interest. It was found that **76** was converted into a mixture of adenosine-5'-carboxaldehydes (vide supra),[171] which was confirmed by comparison with products of chemical synthesis.[172,229,244] Debenzoylation of the 6-*N*-benzoyl-2',3'-*O*-isopropylideneadenosine-5'-carboxaldehyde 5'-(1,3-diphenylimidazolidine) derivative[243b] **104** (Scheme 22) and treatment of the resulting **105** with aqueous trifluoroacetic acid gave a mixture of Ado 5'-carboxaldehyde (**106**) and its 4'-epimer (9-(α-L-lyxofuranosyl)Ade-5'-carboxaldehyde (**109**)) (~5:1).[172] NMR analysis indicated that the hydrate forms (e.g., **107**) predominated in aqueous solution. Epimerization at C4' presumably occurred via the hydroxy enol ethers **108**. Mild acid hydrolysis of the mixed methoxy/methylthio acetals **86** and HPLC purification also gave the Ado 5'-carboxaldehyde hydrate **107**.[172]

Since a 5'-*O*-methyl derivative **83** of the putative hydroxy enol ethers **108** inactivated AdoHcy hydrolase,[178] other stable derivatives of Ado 5'-carboxaldehyde were explored. Oximes (e.g., **113**, Scheme 23) that would be expected to generate the same aldehyde species upon hydrolysis by the enzyme were targeted. Treatment of the protected Ado 5'-carboxaldehyde **111** (prepared from the 1,3-diphenylimidazolidine derivative **104**[243b]) with hydroxylamine hydrochloride in pyridine afforded a diastereomeric mixture of oximes **112** (*E/Z*, ~2.3:1).[244] Deprotection gave the Ado 5'-carboxaldehyde oximes **113** in good yield, but separation of the *E* and *Z* isomers was difficult. Oximes **113** (*E/Z*, 4.5:1) were obtained from 6-*N*-benzoyl-2',3'-*O*-isopropylideneadenosine **110** in four steps (67%) without

Scheme 23.

Ts = 4(CH₃)C₆H₄SO₂; X = F, Cl, Br, I
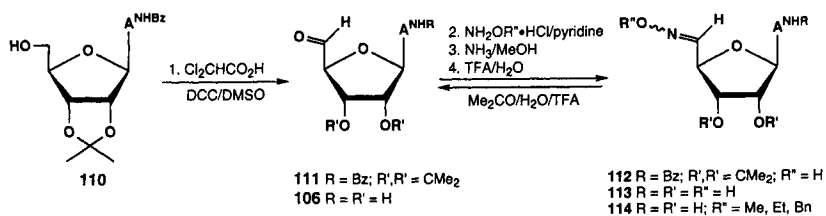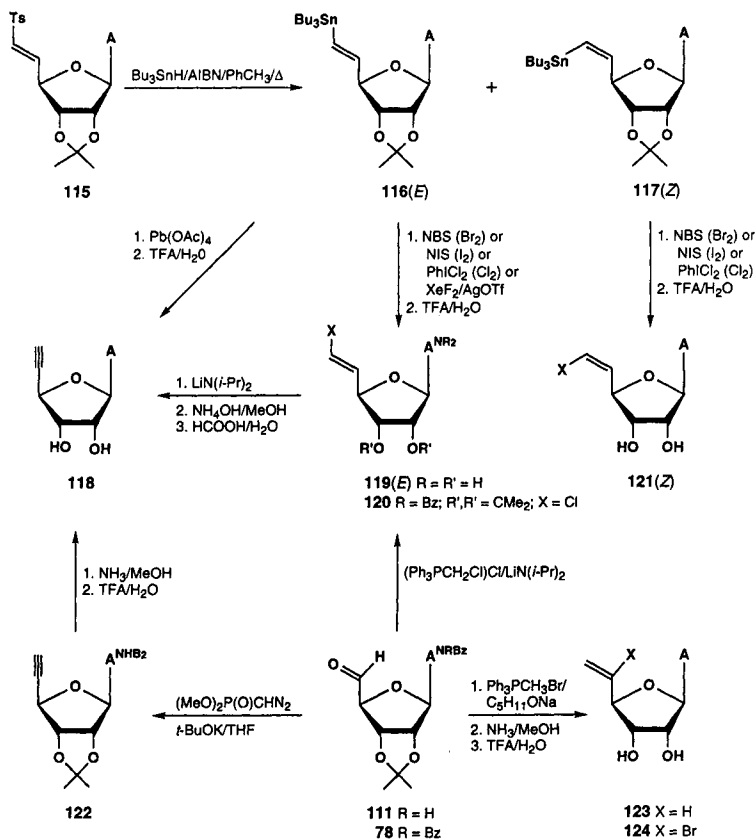
Scheme 24.

isolation and purification of intermediates (except aqueous work-up after step 2). Three *O*-alkyl oxime derivatives **114** also were prepared.[244]

The unprotected *E/Z* oximes **113** were converted to Ado 5′-carboxaldehyde **106** by gentle hydrolysis in aqueous acetone containing trifluoroacetic acid.[244] The 5′-carboxaldehydes of 2′- and 3′-deoxyadenosine and 9-(β-D-arabinofurano-syl)Ade also were synthesized and their oxime derivatives were prepared.[244] Protected oxime **112** has been *O*-tosylated and converted to the 4′-cyano deriva-tive,[245] which is the 6′-aza analog of the 4′-acetylenic compound **118** (Scheme 24).

G. Stereochemically Controlled Synthesis of 6′-Halovinyl Homoadenosine Analogs

Chain extensions and other carbon-carbon bond-forming reactions at C5′ of nucleosides have generally involved oxidation to 5′-aldehydes and Wittig-type reactions with electronegatively stabilized ylides.[243a] Direct introduction of methylene groups at C5′ has met with limited success, presumably owing to instability of the 5′-aldehydes under the strongly basic conditions employed for generation of unstabilized Wittig reagents. However, such a synthesis of 5′-deoxy-5′-methyleneadenosine **123** from **111** was reported recently.[246] Similar treatment of a protected uridine-5′-carboxaldehyde with a (dibromomethylene)triphenylphos-phorane species afforded the 5′-(dibromomethylene)-5′-deoxyuridine derivative, which underwent elimination to afford the 4′-ethynyl (acetylenic) product.[247] Treatment of the protected Ado 5′-carboxaldehyde **78** with (chloromethyl)-triphenylphosphonium chloride and lithium diisopropylamide was reported to give a protected 5′-(chloromethylene)-5′-deoxy analog **120** (stereochemistry not indi-cated), which was converted to the 4′-acetylenic derivative and deprotected to give **118**.[245] Acetylenic derivative **122** also was prepared from **111** by a modified Wittig reaction with dimethyl diazomethylphosphonate and deprotected to give **118**.[248]

A general procedure for the synthesis of 6′(*E* and *Z*)-halohomovinyl nucleoside analogs **119**(*E*) and **121**(*Z*) from protected 5′-carboxaldehydes has been developed recently. Successive Moffatt oxidation[243b] of 2′,3′-*O*-isopropylideneadenosine (and uridine) and treatment of the crude 5′-aldehydes with the stabilized ylid (*p*-tolue-nesulfonylmethylene)triphenylphosphorane afforded the 6′(*E*)-tosylvinyl ho-moadenosine **115**[246] (and its uracil analog)[249] in high yields. Radical-mediated chain extension at C4′ with phenyl vinyl sulfone followed by thermolysis of a derived 6′-(α-pyridyl) sulfoxide had given an alternative synthesis of the 6′-vinyl sulfone homonucleoside **115**.[250] Radical-mediated stannyldetosylation (tributyl-stannane/α,α′-azobis(isobutyronitrile)/toluene/Δ)[251] of **115** gave a mixture of the vinyl 6′-stannanes **116**(*E*) and **117**(*Z*) (~61%; *E/Z*, ~4.2:1)[175] (uracil analogs: 87%; *E/Z*, ~2.8:1).[252] Essentially quantitative and stereospecific halogenodestannyla-tions of the separated **116**(*E*) and **117**(*Z*) isomers occurred with bromine or iodine (or the respective *N*-halosuccinimides) to give 6′-bromo(or iodo)homovinyl Ados

119(*E*) and **121**(*Z*) (X = Br or I) after deprotection.[175] Chlorine converted **116**(*E*) to the 6′-chloro derivative **119**(*E*) plus minor quantities of **121**(*Z*) (X = Cl).

Treatment of **116**(*E*) with XeF_2/silver triflate,[253] deprotection, and HPLC separation gave a mixture of homovinyl 6′-fluoride **119**(*E*) (X = F) and the protiodestannylated **123** (~3:1) in good yield.[175] Oxidative destannylation of **116**(*E*)/**117**(*Z*) with lead tetraacetate, or protiodestannylation with aqueous trifluoroacetic acid or ammonium fluoride in ethanol, and removal of the isopropylidene group gave acetylenic analog **118**, or the 5′-deoxy-5′-methylene compound **123**, respectively, in high yields.[175,252] Treatment of the 2′,3′-*O*-isopropylidene derivative of the 5′-methylene compound **123** with bromine gave the 5′,6′-dibromo diastereomers, which were dehydrobrominated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and deprotected to give 5′-bromo-5′-deoxy-5′-methyleneadenosine (**124**), the regioisomer of **119**(*E*)/**121**(*Z*) (X = Br).[175]

H. Miscellaneous 5′-Modified Adenosine Derivatives

The acetylenic derivative 5′-deoxy-5′-methynyladenosine[248] (**118**), as well as 5′-azido-5′-deoxyadenosine[254] and 5′-cyano-5′-deoxyadenosine,[254] was found to inactivate AdoHcy hydrolase. In addition, Ado 5′-carboxaldehyde (per se[172] or upon enzymatic generation from the prodrug 5′(*Z*)-fluoromethyleneadenosine[171] (**76**)) and homoadenosine-6′-carboxaldehyde (or a spontaneous decomposition product generated therefrom during incubation with the 6′-halogenovinyl homoadenosine derivatives **119**(*E*) and **121**(*Z*))[173,174] function as potent inactivators of this enzyme. Therefore, the synthesis of 5′-modified analogs derived from Ado 5′-carboxylic acid and their evaluation as putative enzyme inactivators were undertaken.[255]

Oxidation of 2′,3′-*O*-isopropylideneadenosine with potassium permanganate[256] gave the protected adenosine-5′-carboxylic acid **125a** (Scheme 25), which was treated with diazomethane to give the methyl ester **126a** (R′ = CH_3). The 5′-carboxamide and *N*-substituted amides **127a** were prepared by treatment of **126a** (R′ = CH_3) with ammonia and primary amines.[255] Acid **125a** was activated with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and coupled with aniline to give *N*-phenyl carboxamide **128a**. Several Ado 5′-carboxylic acid ester[257] and amide[258] derivatives had been prepared by conversion of **125a** to the acid chloride **129a** and treatment of **129a** with alcohols and amines. *N*,*N*-Diethylamide **130a** was prepared by this procedure.[255] Acid-catalyzed removal of the isopropylidene group gave the esters **126b** and amides **127b**, **128b**, and **130b**. Studies of these compounds with AdoHcy hydrolase are of enhanced interest since the recent demonstration (vide supra) that this enzyme can catalyze the "hydrolysis" of 6′(*E*)-halohomovinyl Ado analogs (especially the 6′-fluoro compound **119**(*E*) (X = F)) independently of its C3′ oxidative activity.[173,174]

It was reported recently that 9-(α-L-lyxofuranosyl)Ade (the 4′-epimer of Ado) functions as an alternative substrate of AdoHcy hydrolase.[259] This supports the finding that **109**, the 4′-epimer of Ado 5-carboxaldehyde, is a potent inhibitor of

Series a: R,R = CMe$_2$ R' = H, Me, Et, Bu, Bn, ClCH$_2$CH$_2$
       b: R = H
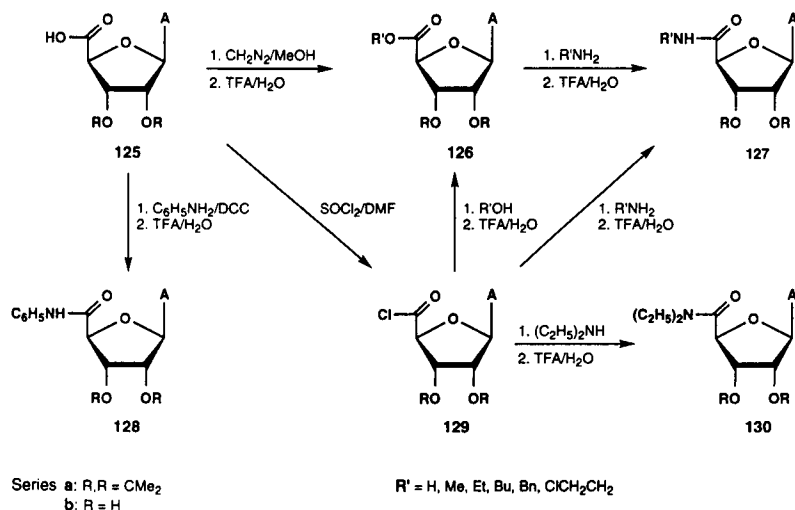
Scheme 25.

the enzyme.[172] These results indicate a rather broad spatial leniency within the active site of AdoHcy hydrolase in the region occupied by C4′ substituents of alternative substrates. To further explore this hypothesis, 9-(α-L-lyxofurano-syl)Ade (**137**, Scheme 26), its 5′-carboxaldehyde **109**, and oxime derivatives **135** were prepared.[244] D-Gulonic-γ-lactone was converted to its 2,3;5,6-di-*O*-iso-
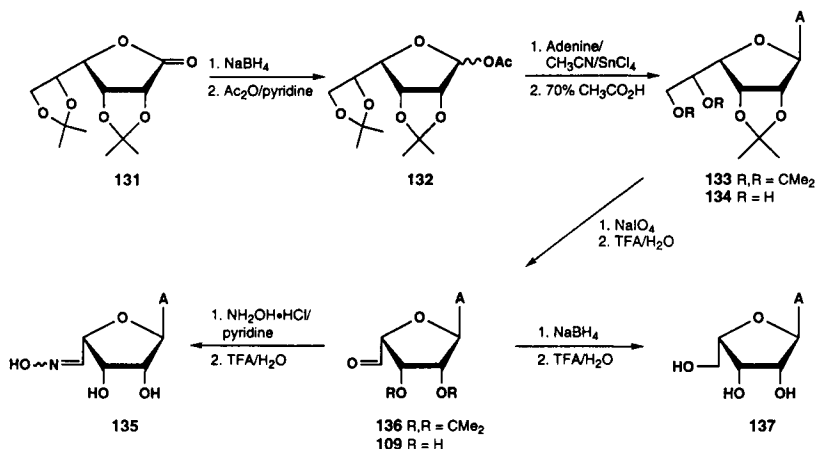
Scheme 26.

propylidene derivative **131** as described.[260] Reduction and acetylation gave the anomers **132** (~9:1), which were coupled with Ade in acetonitrile with tin(IV) chloride catalysis[261] to give the protected 9-(β-D-gulofuranosyl)Ade **133** and its α-anomer (~40%, ~3:1). Selective removal of the 5′,6′-*O*-isopropylidene group and periodate cleavage of the resulting diol in **134** gave the 2′,3′-*O*-isopropylidene-protected 5′-carboxaldehyde **136**. Reduction in situ with sodium borohydride and deprotection gave 9-(α-L-lyxofuranosyl)adenine **137**.[244,260] Aldehyde **136** was deprotected to give **109**.[244] The kinetics of inactivation of AdoHcy hydrolase by this directly synthetic sample of **109** and that produced by equilibration of Ado 5′-carboxaldehyde (**106**, Scheme 22) were identical within experimental error. Treatment of **136** with hydroxylamine hydrochloride in pyridine followed by deprotection gave the somewhat unstable oximes **135** in low yield.[244]

I. 2′-Deoxy-2′-methyleneadenosine and Bis(methylene) Analogs

Treatment of 2′-keto-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxan-1,3-diyl)Ado (**138**, Scheme 27) with methylenetriphenylphosphorane gave the silylated 2′-methylene derivative **140**,[262] which was deprotected to give 2′-deoxy-2′-methyleneAdo (**142**).[262–264] Since it was known[26] that the 4′-methylene analog of Ado, 9-(5-deoxy-β-D-erythro-pent-4-enofuranosyl)Ade, is an alternative substrate of AdoHcy hydrolase, it was reasoned that its regioisomer **142** might also undergo enzymatic oxidation of the allylic 3′-hydroxyl function and cause mechanism-
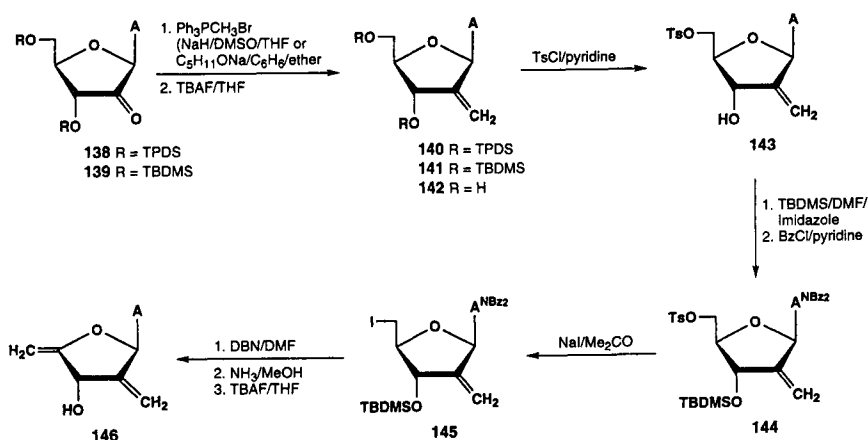
TBDMS: *tert*-butyldimethylsilyl; TPDS: 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl

Scheme 27.
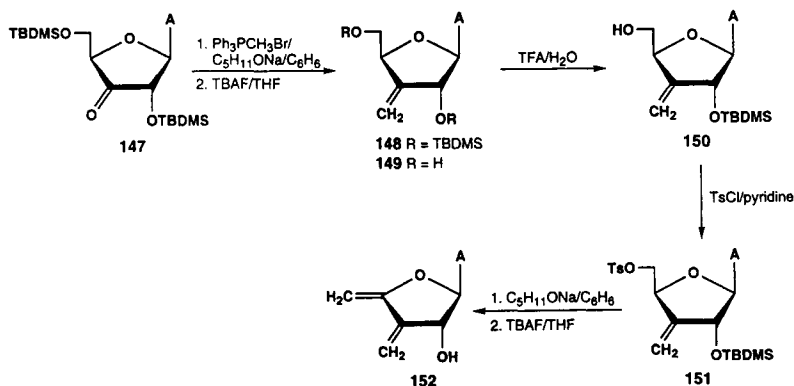
Scheme 28.

based inactivation.[263,264] Indeed, **142** caused inactivation of AdoHcy hydrolase, and potent anticancer activity was found with its 2′-deoxy-2′-methylenecytidine analog.[264,265] The bis(methylene)Ado analog **152**, an "approximate isostere" of the 3′-keto-4′-methylene intermediate in the processing of Ado or AdoHcy by AdoHcy hydrolase,[264] and its regioisomer **146** also were synthesized.[263]

Oxidation of the 3′,5′- and 2′,5′-O-bis(*tert*-butyldimethylsilyl)Ados with chromium trioxide/pyridine/acetic anhydride[266a] or the Dess-Martin 12-I-5 periodinane reagent[266b] gave the respective 2′-keto **139** and 3′-keto **147** (Scheme 28) derivatives. Treatment of **139** with methyltriphenylphosphonium bromide and sodium 2-methyl-2-butoxide in diethyl ether/benzene gave the 2′-deoxy-2′-methylene derivative **141** (~35%,[263] ~90% for the uridine analog[267]), which was deprotected to give 2′-deoxy-2′-methyleneAdo **142**. Regioselective O5′-tosylation gave **143**, which was silylated at O3′ and dibenzoylated at N6 (to prevent intramolecular attack of N3 at C5′) to give **144**.[263] Displacement of the 5′-tosylate group with iodide gave **145**, which underwent dehydrohalogenation with 1,5-diazabicyclo[4.3.0]non-4-ene (DBN). Deprotection gave the 2′,5′-bis(methylene) compound **146**, which was purified by HPLC.[263]

Analogous treatment of the 3′-keto derivative **147** gave the 3′-deoxy-3′-methylene compound **148**, which was deprotected to give **149**.[263] Selective removal of the silyl group from O5′ of **148** with aqueous trifluoroacetic acid[268] gave 2′-O-*tert*-butyldimethylsilyl-3′-deoxy-3′-methyleneAdo (**150**), which was tosylated at O5′ to give **151** and subjected to base-promoted elimination with sodium 2-methyl-2-butoxide. Deprotection gave 4′,5′-didehydro-3′,5′-dideoxy-3′-methyleneAdo (**152**).[263] These methylene-nucleoside congeners caused weak inhibition of AdoHcy hydrolase.[264]

ACKNOWLEDGMENTS

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CARBOCYCLIC NUCLEOSIDES

Victor E. Marquez

I. INTRODUCTION

The field of carbocyclic nucleoside chemistry experienced a phenomenal growth in the years following the completion of our first review in 1986.[1] Today, because of the enormous amount of information available, it would be difficult to combine in one review a comprehensive essay encompassing both the chemical syntheses and biological properties of carbocyclic nucleosides. Fortunately, two excellent reviews dealing primarily with the strategies for chemical syntheses have appeared recently. The first one, by Borthwick and Biggadike,[2] addresses the issue of synthetic approaches to chiral carbocyclic nucleosides, whereas the second, by Agrofoglio et al.,[3] is an encyclopedic account of all strategic syntheses reported up to the end of 1993. Earlier in 1988, a publication by Roberts et al.[4] presented an account on the synthesis of some antiviral carbocyclic nucleosides, and the activity and mechanism of action of several antiherpetic agents, including some carbocyclic analogs, have been recently reviewed by De Clercq.[5] The present review intends to approach the subject from the point of view of structure–activity, with almost an exclusive emphasis on antiviral effects. It is hoped that the manner in which the subcategories have been selected will help understanding of the evolution of the concept of a stable "carba-nucleoside isostere" and how it continues to be applied in the development of newer classes of compounds. In addition, it is hoped that the selected format will allow the reader to discern if a limit has been reached, or if there is still room for additional growth that will invigorate the field again in the same manner as it was energized by the discovery of the neplanocins,[6,7] and the creation of the cyclobutyl analogs of oxetanocin A.[8] I firmly believe that creativity, be it from nature or from the minds of synthetic organic chemists, has no limits, and the few structures selected below represent a testimony to this conviction (Figure 1). Briefly, the synthetic cytosine analog of neplanocin A, cyclopentenyl-cytosine (CPE-C), which was developed as a potent antitumor and antiviral agent, is in phase I clinical trials[9]; truncated forms of neplanocin A, exemplified by the structure of the dihydroxycyclopentenyl derivative, have improved antiviral selectivity and reduced cytotoxicity[10]; the displacement of the double bond to a different position relative to the neplanocins has given rise to the potent and specific antiretroviral agent carbovir[11]; the introduction of fluorine in the structure of the carbocyclic analog of 2'-deoxyguanosine (carba-2'dG) has elevated the antiviral activity of this drug to an unprecedented level[12]; the carbocyclic oxetanocins, particularly the guanine analog carba-oxetanocin G, have shown superb antiher-
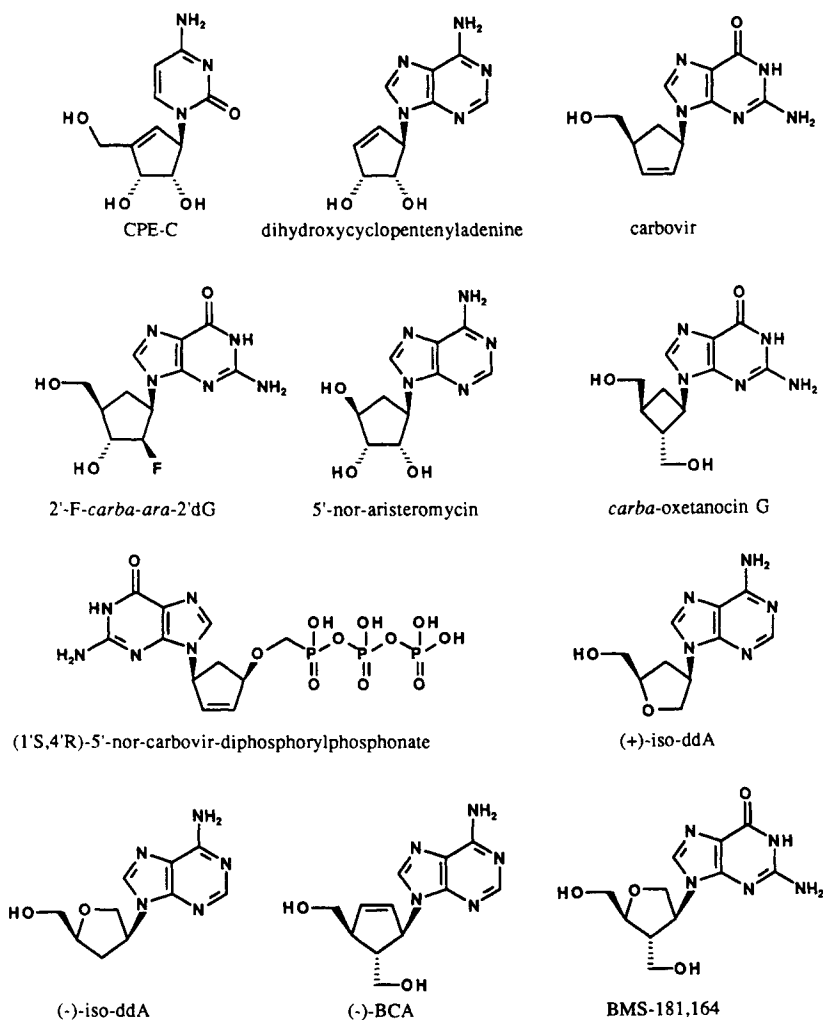
Figure 1. Chemical structures of important *carba*-nucleosides.

petic activity that complements that of its oxetanosyl counterpart[13]; 5'-nor-aris-teromycin has provided a new template for the development of new agents against cytomegalovirus infection[14]; the diphosphorylphosphonate of 5'-nor-carbovir, with the unnatural configuration, is a more potent inhibitor of HIV reverse transcriptase than carbovir triphosphate itself[15]; the homomethylene derivative of carbocyclic oxetanocin A ((−)-BCA) is a hybrid structure with excellent antiretroviral activity[16]; and finally, the development of (+)- and (−)-iso-ddA as novel antiretroviral agents,[17,18] plus the new transposed antiherpetic guanine isonucleoside BMS-

181,164,[19] are only a fraction of some new and fascinating structures that have been synthesized in recent years.

The original underlying concept for the synthesis of carbocyclic nucleosides was to seek a stable C-N bond to replace the hydrolytically and enzymatically scissile glycosylic bond, while causing minimal structural disturbances. However, with relatively few exceptions, the activities of most conventional carbocyclic nucleosides have been poorer than those of the corresponding ribosides. Since the conformation of the five-membered ring is believed to play a critical role in modulating biological activity, a simple comparison between a conventional nucleoside and its carbocyclic counterpart should convince us that the structural changes following the removal of the oxygen are indeed significant. Thus, a comparison between the sugar ring puckering of thymidine and carba-thymidine is very eloquent (Figure 2). The loss of the furan oxygen in carba-nucleosides abolishes the anomeric effect, as well as important gauche interactions between the oxygen and the 2'- and 3'-hydroxyl groups. In nucleosides, the combined effect of these important interactions forces the sugar ring into two preferred forms of puckering in the pseudorotational cycle[20]: (1) a northern conformation neighboring a 2'-exo/3'-endo form of ring puckering, and (2) a southern conformation neighboring the 2'-endo/3'-exo form (as in thymidine in Figure 2). In solution, the conformation of any nucleoside is represented by an equilibrium between these two extremes, and the direction of this equilibrium is often determined by the interplay of the above-mentioned forces. In the solid state, generally only one of the two typical solution conformations is present, and its selection is usually determined by specific crystal packing forces. Similarly, when a nucleoside or nucleotide binds to its target enzyme, only one form is present in the drug–receptor complex. While the energy gap between northern and southern conformations is in the range of 3–4 kcal/mol, one must remember that a favorable energy difference of as low as 1.3 kcal/mol can result in a tenfold decrease in the binding constant (tenfold increase
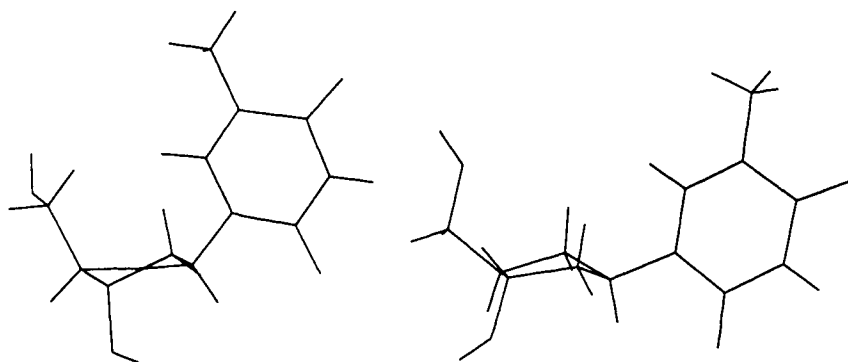

Figure 2. Crystal structures of thymidine (left) and carba-thymidine (right).

in affinity) of a ligand with its receptor.[21] This means that differences in binding could vary between 100- to 1000-fold, depending on the preferred conformation of the nucleoside ligand. Since the cyclopentane ring in carbocyclic nucleosides exists in an unusual 1′-*exo* form (as in *carba*-thymidine in Figure 2), which is relatively far from the conventional 2′-*exo*/3′-*endo* or 2′-*endo*/3′-*exo* forms, this conformational difference could explain why certain carbocyclic nucleosides are generally less effective than their nucleoside counterparts. Recently, however, with some of the nontraditional carbocyclic nucleosides, chemists have been able to compensate for this deviation by selecting novel templates that allow the hydroxyl and nucleobase pharmacophores to mimic the orientation found in one of the natural nucleoside conformers.

In addition to the conformational differences just discussed, the removal of the oxygen represents a dramatic change in terms of stereoelectronic effects. For example, the basicity of the aglycone heterocycle in *carba*-nucleosides is expected to increase and to facilitate protonation at physiological pH relative to the nucleoside.[22] An additional complicating factor is that in most cases nucleosides and their carbocyclic analogs constitute prodrug forms that must be activated to the requisite mono-, di-, or triphosphate anabolites. This means that, at each stage, the drug is interacting with a different set of activating enzymes before it reaches the intended biological target.

To facilitate this presentation, the nomenclature originally proposed by Griengl et al.[23] for carbocyclic nucleosides has been followed, except for cases where the traditional names are more easily recognized. With this system, the replacement of the ring oxygen is symbolized by the term *carba*, which is also used as a prefix when referring to carbocyclic nucleoside analogs of traditional compounds. Otherwise, the term *carba* will be incorporated into the name of the compound, which will be done according to the carbohydrate nomenclature. In this manner, the use of the conventional numbering system for nucleosides is preserved, and the number for the extra carbon atom is designated as 1′a (Figure 3).
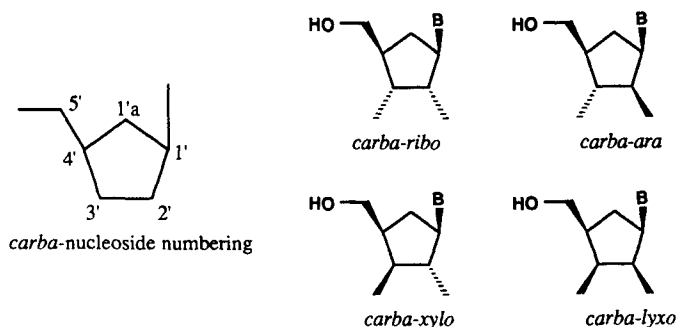

carba-nucleoside numbering

carba-ribo

carba-ara

carba-xylo

carba-lyxo

Figure 3. Numbering system and common carbocyclic templates.

Finally, the organization of the various categories of compounds in this chapter was done strictly for didactical purposes. Inevitably, with some of the more complex structures some redundancy and overlap may occur.

II. *CARBA*-NUCLEOSIDES

This section includes carbocyclic nucleosides containing a saturated cyclopentane ring with substituents on the pseudofuranose ring except fluorine.

A. Purines

In recent years, *carba*-2′-deoxyguanosine (*carba*-2′dG; Figure 4) has emerged as one of the most impressive compounds of this group because of its potent and selective activity against hepatitis B virus (HBV). HBV infection and its progression to hepatocellular carcinoma represent a major health hazard in the world today. (±)-*carba*-2′-dG, which was synthesized more than 10 years ago by Shealy et al.,[24] produced almost complete disappearance of replicating HBV in vitro as measured by DNA polymerase activity, extracellular DNA, and episomal intracellular DNA.[25] Other well-established antiviral agents, such as acyclovir and AZT, proved to be inefective under the same conditions.[25] *carba*-2′dG did not affect the integrated HBV DNAs, and hence cessation of treatment resulted in a return of HBV virus to both intra- and extracellular populations. However, as relatively few natural HBV infections result in genomic integration, *carba*-2′dG is still very effective as it can eliminate HBV-specific episomal DNAs, which are the precursors to the integrated DNAs.[25] In infected ducks, the drug efficiently reduces the number of infected hepatocytes,[26] where an accelerated hepatocyte turnover induced by *carba*-2′dG appears to be essential for clearing the virus.[27] Mechanistically, radioactive *carba*-2′dG is directly phosphorylated and incorporated exclusively into DNA, functioning as a competitive inhibitor of dGTP.[28] With HBV polymerase and DNA polymerase δ, the K_m values for dGTP are similar. However, the K_i of *carba*-2′dGTP for the HBV polymerase is six times lower. This means that at low concentrations
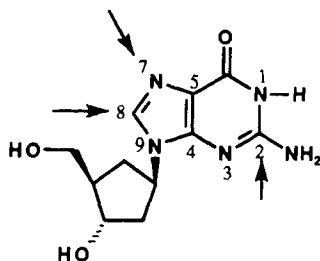

Figure 4. *Carba*-2′-deoxyguanosine (*carba*-2′dG) and common sites of alterations.

carba-2′dGTP is capable of preferentially inhibiting the viral polymerase.[28] The compound, however, does not appear to function as a DNA chain terminator.[28] The efficacy of the drug in experimentally infected ducks with hepatitis virus (DHBV) could be also attributed to the very long half-life of its phosphorylated form, which makes *carba*-2′dG one of the most effective nucleosides against this virus display-ing long-acting effects, even after oral administration.[29] *carba*-2′dG is also a very effective antiherpetic agent active against herpes simplex 1 and 2 (HSV-1 and HSV-2). The compound functions as a good substrate for the virus-coded thymidine kinase (TK) and a poorer substrate for cellular phosphorylating enzymes.[30] Despite this difference, the compound still has significant activity against the HSV-1 TK⁻ strain.[30] Antiviral activity is associated primarily with the D-enantiomer ((+)-*carba*-2′dG), although the L-enantiomer has lower but significant activity.[30] A partially purified preparation of HSV-1 TK, which contained cellular nucleotide kinase activities, converted (+)-*carba*-2′dG almost exclusively to the triphosphate, and (−)-*carba*-2′dG almost exclusively to the monophosphate. In uninfected CEM cells, deoxycytidine (dCyd) kinase seems to be the enzyme responsible for the phospho-rylation of both enantiomers; thus, both HSV-1 TK and cellular dCyd kinase do not appear to show selectivity for either enantiomer. However, the ensuing cellular nucleotide kinases are able to discriminate between them.[31] Also, since HBV does not encode for a nucleoside kinase, initial phosphorylation of *carba*-2′dG must be catalyzed by cellular kinases, principally dCyd kinase.[31] The synthesis of pure (+)-*carba*-2′dG has been achieved, and its potency against HSV-1-infected cells was roughly half of the corresponding value for the racemate.[32] Resolution of (+)-*carba*-2′dG was also accomplished through the action of adenosine deaminase (ADA) on the 2,6-diamino analog.[33] In other studies with HSV in Hep-2 cells, it could be demonstrated that *carba*-2′dG was incorporated into both HSV and host cell DNA, primarily into internal positions of the DNAs.[34] In this case, *carba*-2′dGTP behaved as a competitive inhibitor against dGTP for HSV DNA polymerase and human polymerase α, whereas DNA polymerases β or γ were not affected.[34] Interestingly, although (+)-*carba*-2′dG is structurally related to carbovir (Section VB), and both are efficiently phosphorylated to the 5′-triphosphate in CEM cells to a similar degree, the compound has no activity against the human immunodefi-ciency virus (HIV).[35] (+)-*carba*-2′dGTP did inhibit HIV reverse transcriptase (RT), but the K_i/K_m ratio was 20-fold greater than that for (±)-carbovir-TP, indicating that a much greater intracellular production of (+)-*carba*-2′dGTP would be needed to inhibit HIV replication to the same degree as with (±)-carbovir-TP.[35] In addition, (±)-carbovir-TP functions as an absolute chain terminator, whereas (+)-*carba*-2′dGTP allows for some additional extension of the DNA chain after incorpora-tion.[35] The triphosphate of the opposite enantiomer ((−)-*carba*-2′dGTP) was very inefficient, indicating that HIV-RT is capable of discriminating between the two enantiomeric forms.[35] One must conclude, therefore, that substitution of a ribose with a cyclopentane in nucleotides is not well tolerated by HIV-1 RT. Structural changes on the base, as well as on the carbocyclic template of *carba*-2′dG, resulted

in compounds with varying degrees of antiviral activity. The 3'-deoxy analog, carba-3'dG, and other 2-amino-6-substituted purine and 8-azapurine analogs were synthesized and investigated. carba-3'dG and the 2-amino-6-chloro analog exhibited antiviral activity against HSV-1, and to a lesser extent against HSV-2.[36] However, antiviral activity was either equal to that of ara-A, or less than that of acyclovir.[36] This is an interesting finding since most carba-3'-deoxy analogs are usually devoid of biological activity. The carba-ara configuration was investigated much earlier, and only the 2,6-diaminopurine analog displayed activity against HSV-1.[37] With the carba-xylo configuration, both guanine and 8-azaguanine analogs were effective not only against HSV-1 and HSV-2, but also against human cytomegalovirus (HCMV) and varicella zoster virus (VZV).[38] Investigations with the carba-lyxo configuration indicated that the guanine analog had significant activity against HSV-1 and against HCMV, comparable to that of the carba-xylo analog.[39] carba-Nucleosides of 7-deazaguanine with carba-ribo, carba-ara, and carba-lyxo configurations were also synthesized.[40] The carba-ara and carba-lyxo configurations led to moderately active compounds against HSV-1 and HSV-2, with the carba-ara being more potent than the carba-lyxo analog.[40] These compounds appear to be more effective against HSV-2, which is contrary to the norm, and the carba-ara analog even showed activity in mice infected with HSV-2.[40] Substitution on the purine ring as in the 2,6-diamino analog, or replacing the 3'-hydroxyl function by an azido group in carba-ara-7-deazaguanosine, abolished completely the anti-HSV activity.[41]

The other two major active structures belonging to this group are aristeromycin (carba-adenosine; Figure 5) and carba-ara-adenosine (cyclaradine), plus some of their deaza analogs. Synthetic (±)-aristeromycin can be resolved through the selective degradation of its monophosphate ((±)-C-AMP) by 5'-ribonucleotide phosphohydrolase, which hydrolyzes selectively the (−)-enantiomer. The unnatural (+)-enantiomer appears devoid of antiviral activity.[42] Aristeromycin, carba-3-deaza-adenosine, carba-8-aza-adenosine, and carba-N6-methyladenosine were all good inhibitors of (S)-adenosylhomocysteine hydrolase (S-AdoHcy-ase), and this activity correlated well with their antiviral effect against vaccinia virus (VV). The
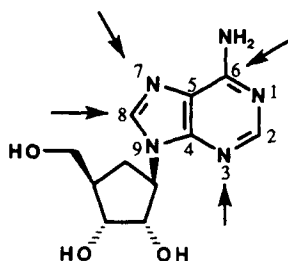

Figure 5. Aristeromycin and common sites of alterations.

most potent compounds, aristeromycin and *carba*-3-deaza-adenosine, functioned as reversible competitive inhibitors of the enzyme, whereas the resulting 2',3'-dialdehydes, obtained by the periodate oxidation of the corresponding carbocyclic nucleosides, were uniformly active and behaved as irreversible inhibitors of the enzyme.[43] *carba*-3-Deaza-adenosine was effective against respiratory syncytial virus (RSV) and parainfluenza 3 virus (PIV3) infections in tissue culture.[44] RSV and parainfluenza virus are the leading cause of serious respiratory tract infections in children under 2 years of age. Therefore, the need for an effective antiviral agent is significant since no vaccines are currently available. Furthermore, ribavirin, the only current therapy approved for use against RSV, has to be delivered in aerosol form. *carba*-3-Deaza-adenosine demonstrated better activity than ribavirin in cotton rats, reducing significantly pulmonary RSV and PIV3 titers.[44]

Aristeromycin M, which corresponds to 5'-deoxyaristeromycin, was isolated from *Streptomyces citricolor*.[45] It was also synthesized[46] and demonstrated to have a spectrum of activity similar to that of other *S*-AdoHcy-ase inhibitors,[47] such as *carba*-3-deaza-adenosine and neplanocin A (Section VA). However, although similar to *carba*-3-deaza-adenosine against VV and vesicular stomatitis virus (VSV), it was less effective against parainfluenza and RSV.[47] It was also less potent than neplanocin A against TK⁻, VZV, and HCMV, although generally less toxic.

Replacement of the 4'-methyl group of 5'-deoxyaristeromycin by $CH{=}CH_2$, phenyl, and CH_2CH_3 (Figure 6) demonstrated that only the β-stereochemistry was compatible with enzyme inhibition.[48,49] Compounds having CH_3, or vinyl groups, were potent inhibitors of *S*-AdoHcy-ase, better than the equivalent compounds derived from neplanocin A. Among all the substituents, ethyl and phenyl groups were the least effective.[48,49] 5'-Deoxyaristeromycin clearly emerged as the most active compound of this group, showing activity against VV and VSV, albeit with a reduced level of potency relative to the parent aristeromycin. From the standpoint
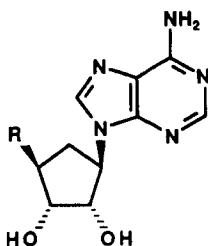
R = CH_3- (5'-deoxyaristeromycin)
R = $CH_2{=}CH$-
R = CH_3CH_2-
R = Ph-

Figure 6.

of structure–activity, it is interesting to note that 5′-deoxyneplanocin was inactive against S-AdoHcy-ase.[48] *carba*-ara-Adenosine (cyclaradine) is well known for its efficacy against herpes infection both in vitro and in vivo. The compound resists deamination by ADA, and it is two to five times more potent than ara-A.[50] In vitro against HSV, (+)-cyclaradine is more potent than ara-A, about as potent as phosphonoformate, and less potent (10 to 200 times) than acyclovir.[50] These large differences in vitro, however, are not reflected in vivo, where cyclaradine is only twofold less potent than acyclovir in the guinea pig model.[50] The drug is phosphorylated by adenosine kinase (AK) and not by dCyd kinase or TK, and thus it shows activity against TK⁻ and acyclovir-resistant mutants.[50] The 5′-methoxyacetate of cyclaradine appears to be a good prodrug for cyclaradine in some animal models.[51] The C4′ epimer of (+)-*carba*-ara-adenosine was synthesized (9-(*carba*-1′-a-β-L-xylofuranosyl)-adenine), but no biological activity was reported.[52]

An important strategy aimed at reducing the toxicity of aristeromycin consisted in removing the hydroxymethyl chain. This strategy, which first proved successful when applied to the neplanocins (Section VA), was also effective in this case. Hence, the corresponding (1′R,2′S,3′R)-9-(2′,3′-dihydroxycyclopentan-1-yl)adenine and the 3-deazaadenine analog exhibited potent antiviral activity against VV with reduced cellular toxicity. These compounds were not only resistant to the action of AK, but also to deamination by ADA.[53] Some other less effective types of molecular changes included the removal of the 6-amino group from aristeromycin, such as in 9-(3′-deoxy-*1′*a-*carba*-β-D-ribofuranosyl)purine (*carba*-3-deoxynebularine), which resulted in an inactive compound against HCMV, VZV, HSV-1, HSV-2, FLU, and HIV in vitro.[54] The incorporation of a large hydrophobic appendage at C2 of the purine ring consisting of either a 4-(1-butyl)phenylamino or a 3,5-dichloro-phenylamino substituent in *carba*-ara-6-oxo- and 6-aminopurine nucleosides led to inactive compounds against HCMV.[55,56] A similar series of chiral C2-substituted 9-(5′-*N*-ethyl-carboxamido-*carba*-1′a-β-D-ribofuranosyl)-6-aminopurines was also reported, but they were only investigated as adenosine receptor agonists.[57] Compounds with additional hydroxyl substituents on the pseudofuranose ring, like 1′a-β-hydroxyaristeromycin, were also
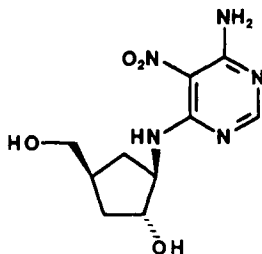

Figure 7. 3′-Deoxy-*carba*-clitocine.

synthesized. [58] These types of hydroxy compounds served as starting materials for some of the fluoro-aristeromycin analogs that will be discussed separately (Section III).

Finally, a group of carbocyclic clitocine analogs, which can be considered as compounds with an incomplete purine ring system, were also investigated. The synthesis of *carba*-clitocine[59] and 3'-deoxy-*carba*-clitocine[60] (Figure 7) was described, and the latter was reported to have activity against HCMV with low cellular toxicity.[61]

B. Pyrimidines

In view of the good antiherpetic activity of *carba*-thymidine[62] and the known antiviral activity of 5-ethyl-2'-deoxyuridine (EDU), the corresponding *carba*-EDU nucleoside was made with the intent to overcome the action of pyrimidine nucleoside phosphorylase on EDU (Figure 8). Although *carba*-EDU and the 5'-ethynyl analog had significant antiviral activity against HSV-1 and HSV-2, they were less effective than EDU and ara-A. Against HSV-1, however, *carba*-EDU was better than *carba*-thymidine.[63] As discussed before, the structure of (+)-*carba*-thymidine shows that the carbocyclic ring adopts an unusual C1'-*exo* ring pucker, which contrasts with the 2'-*endo*/3'-*exo* conformation characteristic of thymidine (see Figure 2).[64]

In addition to (+)-*carba*-thymidine and (−)-*carba*-thymidine, other enantiomerically pure compounds synthesized included *carba*-3'-epi-thymidine, 3'-deoxy-3'-
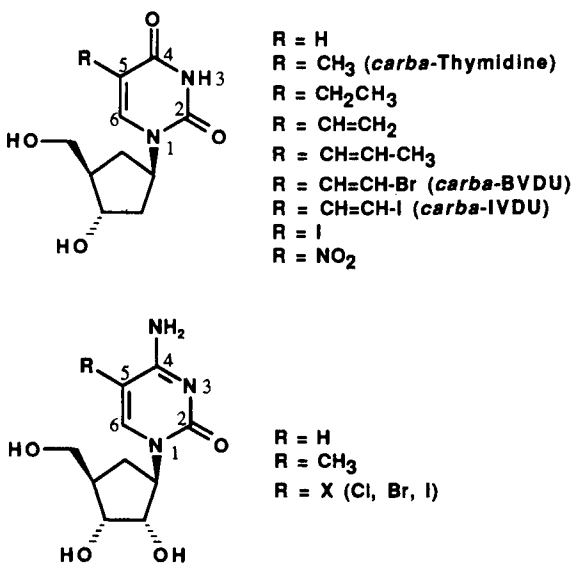
Figure 8. Common *carba*-pyrimidine nucleosides and their numbering system.

azido-1'a-*carba*-thymidine (carbocyclic AZT), and *carba*-1'a-α-methylthymidine. With the exception of (+)-*carba*-thymidine, none of these compounds were active against HSV-1 and HSV-2.[65] Other 1-(2',3'-dideoxy-1'a-*carba*-β-D-ribofuranosyl)-pyrimidine nucleosides synthesized included analogs with 2-thiouracil, 2-thiothymine, cytosine, 5-fluorouracil, and 5-methylcytosine bases. This series, which included the 5-fluoro analog of AZU (5-fluoro-3'-deoxy-3'-azido-1'a-*carba*-uridine), did not produce an active antiherpetic agent.[66,67] The first synthesis of *carba*-AZT (Figure 9) was reported in racemic form along with several compounds that included the 5'-azido-5'-deoxy-analog, the 3' epimer of *carba*-AZT, and the corresponding reduced amino compounds.[68] As antiherpetic agents these compounds showed only moderate to poor activity.[68] The *carba*-2'-azido- and *carba*-2'-amino-2'-deoxycytidine analogs were also synthesized and tested against HSV-1, but only the 2'-amino analog showed moderate antiviral activity.[69] The first synthesis of enantiomerically pure *carba*-AZT was reported in 1987.[70] However, contrary to AZT, the compound was totally devoid of anti-HIV activity. Similarly inactive against HIV was (–)-*carba*-3'-deoxy thymidine.[71]

Among a series of carbocyclic 5-halosubstituted cytosine-nucleosides with ribo-, 2'-deoxy-, ara-, and 3'-deoxy-configurations, the 5-iodo-2'-deoxycytidine analog appeared worthy of further investigation.[72] *carba*-Cytidine (carbodine), 2'-deoxy-carbodine, *carba*-ara-cytidine, and their corresponding 5-iodo derivatives, plus 5-bromo-2'-deoxy-1'a-*carba*-cytidine, were significantly effective against HSV-1 in Vero cells. The role of cytidine deaminase in the activity of these compounds against HSV-1, HSV-2, and influenza was hypothesized. However, deamination to the uracil nucleosides is minimal and the compounds are active per se.[72] A new carbocyclic analog of cytidine, (±)-1'a-*carba*-1'a-β-hydroxycytidine, was synthesized by the direct introduction of the cytosine moiety into an unprotected amino-cyclopentane tetrol. However, no biological activity was reported for this compound.[73]

In view of the potent anti-HIV activity of 4'-azidothymidine,[74] two 4'-azido-carbocyclic nucleosides were synthesized with hydroxyl groups at C1'a (α- and β-anomers; Figure 10). These compounds proved to be inactive against HIV, HSV-1, and HSV-2.[75] More recently, the synthesis of 1'-α-methyl-*carba*-thymidine was reported. Although it was utilized only for the construction of oligodeoxyri-
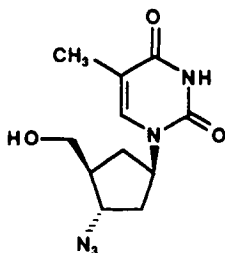

Figure 9. carba-AZT.
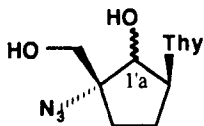
Figure 10. 1'a-Hydroxy-4'-aza-*carba*-thymidine.

bonucleotides, its synthesis opens the way to other related nucleosides that should be investigated for their potential antiviral activity. Conformationally, this compound in solution appears to have a 1'-*exo* conformation in which the 1'-methyl group is in a pseudoaxial disposition.[76]

(*E*)-5-(2-Bromovinyl)-2'-deoxyuridine (BVDU) and (*E*)-5-(2-iodovinyl)-2'-deoxyuridine (IVDU) are recognized to be among the most potent and selective inhibitors of HSV-1 and VZV replication. A negative feature of these drugs, however, is that they are rapidly cleaved by pyrimidine nucleoside phosphorylases (PNPs) and therefore are cleared rapidly from plasma. For this reason, the corresponding carbocyclic nucleosides presented themselves are attractive candidates for circumventing the phosphorolytic cleavage. Racemic *carba*-BVDU and *carba*-IVDU (Figure 8) were initially reported to be almost as active as their riboside counterparts in inhibiting the replication of HSV-1 in primary rabbit kidney cells.[77] Just like the ribosides, they were inhibitory to HSV-2 only at higher concentrations (ca. 100-fold).[77] The specific interaction of *carba*-BVDU and *carba*-IVDU with HSV-1 TK was competitive with respect to thymidine phosphorylation, showing comparable K_i values. Consistent with this mechanism of activation, the compounds were ineffective against the TK⁻ HSV-1 variant and inactive against VV, which codes for a TK different from that induced by HSV-1. Cellular TK was unable to phosphorylate these drugs.[77] Mutant cell lines derived from a murine mammary carcinoma (FM3A/0) that were transformed with the HSV-1 TK gene were strongly inhibited by *carba*-BVDU and *carba*-IVDU at much lower concentrations than those required to inhibit the growth of the parent cell line.[78] However, the nucleosides BVDU and IVDU appeared to be considerably more selective.[78] The mechanism of action of these drugs is probably through the inhibition of thymidylate synthetase, although not exclusively, since the incorporation of these *carba*-nucleosides into the DNA of these mutant cells has been documented.[78] 5-Nitro-2'-deoxy-1-a-*carba*-uridine, which was also targeted against thymidylate synthetase, was found to be a weak inhibitor of this enzyme.[79] Subsequent studies determined that the *carba*-nucleosides of BVDU and IVDU were less potent than the nucleosides. For example, *carba*-IVDU was about tenfold less potent against HSV-1 than the parent IVDU, although it was fourfold less cytotoxic. Mechanistically, although both classes of drugs are activated by viral TK, and both seem to be incorporated into viral and cellular DNAs of HSV-1-infected vero cells, the extent of incorporation of the carbocyclic drugs appears to be less and does not lead to double-strand

DNA breaks.[80] The original expectation that these *carba*-nucleosides by virtue of their lack of degradation by PNPs would be more efficacious did not prove to be correct. In mice, BVDU achieved a better level of protection against HSV-1 infection than racemic (±)-*carba*-BVDU, irrespective of the route of administration. This poor performance in vivo might be related to a difference in pharmacokinetics.[81] (+)-*carba*-BVDU was prepared by using a methodology developed by Griengl et al. for the synthesis of enantiomerically pure carbocyclic nucleosides.[82] The (+)-enantiomer showed a reduced level of potency against HSV-1 and HSV-2 as compared to BVDU, and it was virtually inactive against VV.[83] Interestingly, the level of potency was quite similar to that of the racemate, and surprisingly, the (−)-isomer with the unnatural configuration showed a significant level of antiviral effect 10- to 500-fold less potent than the (+)-isomer.[83] Both (+)- and (−)-enantiomers of *carba*-BVDU displayed strong affinity for HSV-1 kinase and were efficiently phosphorylated. Therefore, they represent examples of chiral molecules in which both enantiomers are markedly active at both the cellular and enzymatic levels.[83] This is in sharp contrast with purine carbocyclic nucleosides where biological activity resides mainly with one enantiomeric form. Indeed, against HSV-2 both (+)- and (−)-*carba*-BVDU showed antiviral activity equal to that of the racemic mixture.[83] In general, the inhibition of HSV-1 TK by BVDU, IDU, (+)-*carba*-BVDU, and (+)-*carba*-IDU showed a linear mixed-type inhibition of HSV-1 TK.[84,85] These herpetic kinases do not appear to be very selective and even tolerate both α- and β-anomers of (±)-*carba*-BVDU.[86]

The enantioselective synthesis of carbocyclic 2′-deoxynucleosides, including *carba*-2′-deoxyuridine and the corresponding (+)-*carba*-IDU, was also reported. In contrast to *carba*-BVDU, the antiviral activity of (+)-*carba*-IDU was roughly half of that shown by the racemic compounds, indicating that activity was associated with the enantiomer corresponding to the natural configuration.[87] Against HSV-1, *carba*-IDU and *carba*-5-propenyl-2′-deoxyuridine were about tenfold less potent than the parent 2′-deoxyribosides, whereas *carba*-3′-deoxy-5-propenyluridine was totally inactive.[88] The inclusion of additional substituents on the template of *carba*-BVDU and *carba*-IDU embodied compounds such as 5-(*E*)-(2-bro-
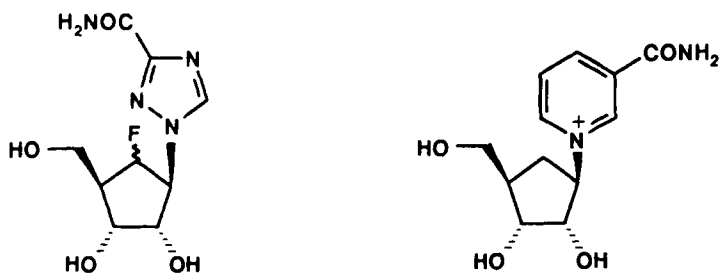

1′-a-fluoro-*carba*-ribavirin   *carba*-nicotinamide riboside

Figure 11.

movinyl)-2',3'-dideoxy-3'-azido-1'a-*carba*-uridine and 5-iodo-2',3'-dideoxy-3'-azido-1'a-*carba*-uridine. These compounds are basically identical to *carba*-BVDU and *carba*-IVDU, but with a 3'-azido group.[89]

The incorporation of the carbocyclic analog of ribavirin in this section might be disputed since some would regard it more as a purine-like nucleoside. The synthesis of *carba*-ribavirin and the 1'a-α- and β-substituted analogs (OH or F) was very informative (Figure 11). In contrast to ribavirin, none of the carbocyclic analogs inhibited the replication of influenza A virus, and none behaved as substrates for AK. At the whole cell level, only *carba*-ribavirin and its 1'a-β-fluoro analog exhibited activity against the enzyme inosine monophosphate dehydrogenase (IMDPH), whereas with the isolated enzyme, the monophosphate of the fluoro-analog was more potent than ribavirin monophosphate itself. Furthermore, ribavirin triphosphate was only a weak inhibitor of viral polymerase and the triphosphate of the fluoro-analog was as potent as ribavirin triphosphate. These results confirmed that inhibition of IMPDH does not confer antiviral activity against influenza.[90]

The synthesis of the carbocyclic analog of nicotinamide riboside was reported, and the compound showed good antibacterial and antifungal activity (Figure 11).[91] The corresponding NAD analog, where the *carba*-nicotinamide replaces nicotinamide riboside, was also synthesized.[92,93]

III. FLUORINATED *CARBA*-NUCLEOSIDES

As mentioned before, the absence of the 4' oxygen in *carba*-nucleosides represents a dramatic change in terms of stereoelectronic effects. Prompted by the described isosteric relationship between oxygen and a fluoromethylene group,[94] the synthesis of fluoro-substituted carbocyclic nucleosides was undertaken, particularly those with fluorines at positions 1'a, 2', and 4', where the inductive effect of the fluorine on the neighboring base and 5'-hydroxyl group was deemed to be relevant.

A. Purines

Studies with 2'-fluoro and 1'a-fluoro-*carba*-nucleosides bearing a purine base led to the discovery of one of the most potent compounds ever found against HSV-1 and HSV-2. 1-(2'-Deoxy-2'-fluoro-1'a-*carba*-β-D-arabinofuranosyl)guanine (2'-β-F-*carba*-2'dG; Figure 12) is more potent than *carba*-2'dG, ara-G (9-β-D-arabino-
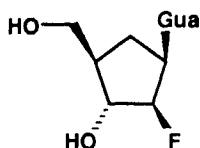

Figure 12. 2'-β-F-*Carba*-2'dG.

furanosylguanine), and acyclovir.[12] An interesting aspect regarding the biological consequences of changing to a carbocyclic moiety in this series is that *carba*-ara-G is inactive against HSV-1.[95] Among the four target compounds originally synthesized were racemic 2'-α- and 2'-β-fluorocarbocyclic guanines and the enantiomerically pure 1'a-α- and 1'a-β-fluorocarbocyclic guanines. The 2'-β-fluoro stereochemistry was associated with a 1400-fold increase in potency relative to the 2'-α-isomer, whereas the opposite trend was observed for the 1'a-isomers. In vivo studies also confirmed that 2'-β-F-*carba*-2'dG was two orders of magnitude more potent than acyclovir against HSV-1, and 70-fold more potent against HSV-2. This compound definitely reversed the trend that carbocyclic versions of antiviral nucleosides are less potent than their nucleoside equivalent.[12] The biological activity of 2'-β-F-*carba*-2'dG was associated only with the (–)-isomer.[96]

The two 1'a-fluoro epimers of 2',3'-dideoxy-1'a-*carba*-nucleosides with guanine and adenine bases showed no activity against HIV in infected cells.[97] Similarly, 2',3'-dideoxy-3'-fluoro-1'a-*carba*-β-D-erythro-pentofuranosyl nucleosides with the identical purine bases were synthesized (Figure 13), but only 9-(2',3'-dideoxy-3'-fluoro-1'a-*carba*-β-D-erythro-pentofuranosyl)guanine showed marked activity against HSV-1.[98] 9-(2',3'-Dideoxy-3'-fluoro-1'a-*carba*-β-D-erythro-pentofuranosyl)adenine was only minimally active against adenovirus type 398 and HIV in H9 cells.[99] Fluorine substitutions at other sites of the carbocyclic ring were also investigated. Placement of the fluorine at C4' did not have the same dramatic effect as in C2'.[100] The rationale behind this type of substitution was to mimic the electronic environment around the functionally important 5'- and 3'-hydroxyl groups in nucleosides with the electronegative fluorine. Both 4'-hydroxy and 4'-fluoro analogs of the potent *carba*-2'dG were prepared in enantiomerically pure form from aristeromycin, and the studies showed that the stereochemistry at C4' was crucial for biological activity. Not surprisingly, the 4'-β-hydroxy analog was only weakly active against HSV-1 and HSV-2, whereas both 4'-α-hydroxy and 4'-α-fluoro derivatives were comparable in activity to acyclovir. Furthermore, these compounds retained the same level of potency of *carba*-2'dG against HSV-2 with the 4'-α-hydroxy compound being less toxic to Vero cells.[101] Compounds with two fluorine atoms in a 2',3'-dideoxy-2',3'-difluoro-1'a-*carba*-ribofuranosyl template, in which both fluorine atoms are below the plane of the ring, were reported, but no biological activity was disclosed.[102]
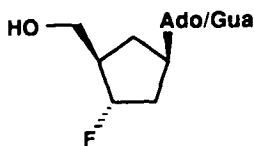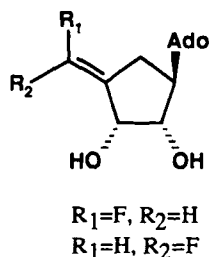

Figure 13.   2',3'-Dideoxy-3'-fluoro-1'a-*carba*-β-D-*erythro*-pentofuranosyl purines.

R$_1$=F, R$_2$=H
R$_1$=H, R$_2$=F

Figure 14.

The other important carbocyclic nucleoside that was subject to extensive substitution studies with fluorine was aristeromycin. Indeed, the first direct introduction of fluorine into a carbocyclic nucleoside was reported with the synthesis of 2′-deoxy-2′-fluoro-*carba*-ara-aristeromycin, which showed potent antiherpetic activity in vitro and in vivo. This compound was at least 10 times more potent than cyclaradine (ara-aristeromycin) against HSV-1 and HSV-2 in vitro, and more potent than acyclovir against HSV-2 in mice.[103]

The activity of C1′a-substituted aristeromycins was also investigated in terms of inhibition of *S*-AdoHcy-ase. The 1′a-β-fluoro epimer showed powerful inhibition of this enzyme, whereas the inverted epimer experienced a 20-fold drop in potency.[104]

Carbocyclic (*E*)- and (*Z*)-4′,5′-didehydro-5′-deoxy-5′-fluoroaristeromycins (Figure 14) were synthesized as targets against the same enzyme, but were less effective than the corresponding ribosyl vinyl fluorides, which function as "prodrugs" of adenosine-5′-carboxyaldehyde. These carbocyclic analogs, on the other hand, possibly function through the formation of 3′-keto derivatives.[105,106]

B. Pyrimidines

The corresponding C1′a-fluoro anomers of *carba*-IDU were synthesized and compared to IDU and (+)-*carba*-IDU.[107] Despite the initial success reported for the 1′a-α-anomer, both 1′a-α- and 1′a-β-fluoro analogs were actually devoid of antiherpetic activity, suggesting that the fluoromethylene group is inferior to the methylene group as a replacement for the ring oxygen of IDU.[108] The solid-state conformation of the 2′-deoxy-1′a-β-fluoro-1′a-*carba*-uridine precursor revealed an unusual O4′-*endo*-conformation (actually a C1′a-*endo* conformation) when compared to 2′-deoxyuridine (C2′-*endo*) in which the fluorine atom is out of the plane of the five-member ring envelope.[108] This is probably due to the strong *gauche* effect interaction between fluorine and the base nitrogen at C1′. Such a change in ring puckering has a marked effect on the spatial disposition of the base and the primary (5′-OH) group, and it might be related to the differences observed in
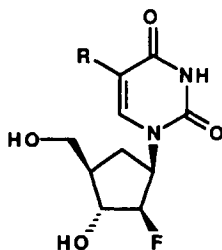
R = I (*carba*-FIAU)
R = CH$_3$ (*carba*-FMAU)

Figure 15.

antiviral activity.[108] Fluorine substitution at C2' was also investigated, and the corresponding carbocyclic analogs of 5-iodo-1-(2'-deoxy-2'-fluoro-β-D-arabino-furanosyl)uridine (*carba*-FIAU) as well as 1-(2'-deoxy-2'-fluoro-β-D-arabinofur-anosyl)thymine (*carba*-FMAU) were synthesized (Figure 15).[109] In keeping with the same trend, the antiviral activity of these compounds against HSV-1 and HSV-2 was greatly reduced relative to that of the parent nucleosides.

Carbocyclic analogs of 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)cytosine and 1-(2'-deoxy-2'-fluoro-β-D-ribofuranosyl)cytosine were only moderately active against HSV-1 and HSV-2.[110] The latter compound is actually an analog of car-bodine, which is known for its activity against the influenza virus. However, neither fluorocytosine carbocycle was active against this virus. The presence of an additional fluorine atom, such as in 2,2'-difluoro-1'a-*carba*-thymidine, destroyed activity.[110]

Application of the same rationale, in which the CHF moiety replaces the O4' oxygen, was investigated in *carba*-AZT with the hope of restoring anti-HIV activity. Two 1'a-fluorocarbocyclic analogs of AZT were synthesized and the enantiomers were resolved.[111,112] Both 1'a-α- and 1'a-β-fluoro anomers of *carba*-AZT, together with the 1'a-α-fluoro anomer of 1-(2',3'-dideoxy-3'-fluoro-1'a-*carba*-β-D-erythro-pentofuranosyl)thymine (1'a-α-F-*carba*-FddT) (Figure 16), were evaluated as antiretroviral agents against HIV. Only the 1'a-α-fluorocarbocyclic isomer of AZT showed weak anti-HIV activity, and its synthetically made triphosphate was about two orders of magnitude less potent than AZT-TP.[113] The conformational shape of this compound is distinctly different from that of AZT in the crystal structure and possibly in solution as well. The carbocyclic ring shows an unusual 1-*exo* conformation, which probably explains its lack of biological activity in this system.[113] The 1'a-α-fluorocarbocyclic analog of AZT, as well as the thiocyanate analog congener, was synthesized in optically active form using an enantiospecific enzyme-catalyzed Baeyer-Villiger type oxidation.[114] The
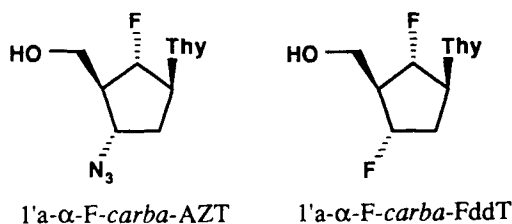
1'a-α-F-*carba*-AZT 1'a-α-F-*carba*-FddT

Figure 16.

stereospecific syntheses of *carba*-FddT and 3'-deoxy-1'a-*carba*-thymidine were also reported.[115,116] A series of 2',3'-dideoxy-1'a-fluoro-*carba*-nucleosides that included thymine and uracil bases were tested for anti-HIV activity. Both 1'a-α and 1'a-β series showed no activity against HIV in infected cells, with the exception of the 1'a-α-fluorothymidine analog, which was only weakly active.[97] Although its synthetic triphosphate behaved as an inhibitor of HIV reverse transcriptase, it was ~100 times less potent than AZT-TP.[97] Finally, the *carba*-analog of FddC, 1-(2',3'-dideoxy-3'-fluoro-1'a-*carba*-β-D-erythro-pentofuranosyl)cytosine, along with other *carba*-dideoxy nucleosides, was evaluated against HSV-1, influenza type A, adenovirus type 3, and VSV, but none were found to be active.[117]

IV. 5'-NOR-*CARBA*-NUCLEOSIDES

The effort to study the effect of removing the C5' carbon on the biological activity of nucleosides would be thwarted by the very nature of the resulting hemiacetal moiety, which is capable of generating mixtures of C4'-epimers, as well as open-chain forms. To overcome this difficulty, this problem was studied with the isosteric *carba*-nucleosides. The first compounds reported in this series were racemic 2'-deoxy analogs with adenine and hypoxanthine bases.[118] The syntheses of the pure enantiomers, (+)-5'-nor-2'-deoxy-aristeromycin and (−)-5'-nor-3'-deoxyaris-teromycin, were reported later.[119] Other analogs included (±)-5'-nor-3'-deoxy-ara-aristeromycin[120] and (±)-5'-nor-1'a-carba-β-D-ribo-furanosyl guanine.[121] Racemic 5'-nor-2'-deoxy-aristeromycin, 9-(5'-nor-2'-deoxy-1'a-*carba*-β-D-ribofurano-syl)guanine, and the corresponding 2,6-diamino analog were inactive against herpes, HCMV, and other viruses, including VV, VSV, and HIV. These compounds also showed very little cytotoxicity, possibly because of their inability to be phosphorylated.[122] On the other hand, (±)-5'-nor-aristeromycin (see Figure 1) was found to have a spectrum of antiviral activity similar to that of other adenosine analogs that target *S*-AdoHcy-ase showing good activity against VV, VSV, parain-fluenza type 3, measles, and RSV. More importantly, the compound represents a new lead for the development of anti-HCMV nucleosides.[14] The pattern of activity

of the 2,6-diamino analog was similar, but it was considerably less potent, and the hypoxanthine and guanine analogs were inactive.[14] Modifications of the base destroyed antiviral activity, as the 7-deaza analog did not inhibit S-AdoHcy-ase and was devoid of any appreciable antiviral activity against a number of DNA and RNA viruses.[123] (–)-5′-Nor-aristeromycin, the active enantiomer having the configuration of the parent aristeromycin, was synthesized, and the method of synthesis was extended to the preparation of (–)-7-deaza-5′-nor-aristeromycin.[124] Synthesis of optically active (–)-5′-nor-aristeromycin was also performed by a lipase-catalyzed acetylation that was performed at the carbocyclic purine stage.[125] (–)-5′-Nor-aristeromycin retained the significant anti-HCMV properties of the racemate and was, on average, tenfold more potent than the L-like (+)-enantiomer in terms of antiviral activity and inhibitory potency against S-AdoHcy-ase. Even though (–)-5′-nor-aristeromycin is deaminated by ADA, the compound appears to be less susceptible to the action of the enzyme, which could be therapeutically advantageous.[126] In addition, the lack of a reactive 5′-OH group prevents enzymatic phosphorylation and avoids the formation of toxic 5′-phosphates. Two derivatives of 5′-nor-aristeromycin in which the C4′-hydroxyl group was inverted (5′-epi-5′-nor-aristeromycin) or replaced by a fluorine were also synthesized (Figure 17).[127] Interestingly, 5′-epi-5′-nor-aristeromycin showed an antiviral activity profile similar to that of 5′-nor-aristeromycin, but it was generally less potent. The compound was particularly active against VV, VSV, parainfluenza-3 virus, and reovirus, and had marked activity against HCMV and VZV. Its antiviral spectrum was similar to that of neplanocin A, a well-known inhibitor of S-AdoHcy-ase.[128] Both C4′-epimers of 5′-nor-2′,3′-dideoxy-1′a-carba-pentofuranosyl guanine, as well as other ring-expanded versions, were synthesized. However, these compounds were devoid of any antiviral activity.[129] Similar cycloalkanol derivatives of guanine, where the base is separated from the carbocyclic ring by a methylene group, were equally ineffective. The latter compounds were designed as constrained analogs of acyclovir, patterned
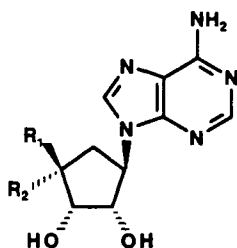
R₁=F, R₂=H
R₁=H, R₂=OH (5'-epi-5'-nor-aaristeromycin)
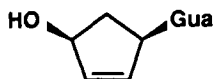
Figure 17.

Figure 18. 5'-Nor-carbovir.

after the cyclohexyl derivative of acyclovir, which behaved as a strong inhibitor of HSV-1 TK.[130] All possible stereoisomers of carbocyclic 2',3'-dideoxy-2',3'-dide-hydro-5'-nor-1'a-*carba*-adenosine were reported,[131,132] as was the 9-(2-cyclopen-tenyl)guanine analog (5'-nor-carbovir; Figure 18).[133] These compounds and related pyrimidine and purine nucleosides can be bis-hydroxylated and thus could serve as starting materials for the preparation of the corresponding 5'-nor-carbocyclic analogs of guanine, adenine, cytosine, and uracil.[133] Finally, carbocyclic nucleo-sides that could be described as 5'-deoxy-5'-nor-aristeromycin and 5'-deoxy-5'-nor-3-deazaaristeromycin are in essence 2',3'-dihydroxycyclopentan-1'-yl nucleosides and were described in Section IIA.[53]

V. CYCLOPENTENE-CONTAINING *CARBA*-NUCLEOSIDES

A. Neplanocin and Related Nucleosides

Purines

Neplanocin A (Figure 19) is the paradigm among the broad-spectrum antiviral agents that function as inhibitors of *S*-AdoHcy-ase. The correlation between the inhibitory capacity of this enzyme and the ensuing antiviral activity, particularly against negative-stranded RNA viruses, has been well established.[134] The inhibition of *S*-AdoHcy-ase by neplanocin A results from its oxidation to the 3'-keto deriva-tive, which occurs with the concomitant reduction of enzyme-bound NAD^+ to
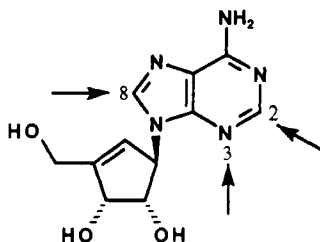
Figure 19. Neplanocin A and common sites of alterations.

NADH. The resulting inactivation of the enzyme is due to the high affinity of the 3'-keto intermediate for the NADH form of the enzyme, which makes it catalytically incompetent.[135] Another important correlation appears to exist between inhibition of this enzyme and activity against the replication of cytomegalovirus (CMV) infection. The level of this correlation, however, is less clear, suggesting that there is an additional unknown underlying mechanism responsible for this activity.[136] In general, a ribose-like cyclopentene moiety appears to be essential for biological activity, since 2'-deoxy-neplanocin A, as well as the corresponding ara-neplanocin A, was reported to be devoid of either cytotoxicity[137] or antiviral activity.[138] The nature of the aglycone is also quite important since the deaminated product, neplanocin D,[138] as well as the guanosine analog, was equally devoid of biological activity.[139] The corresponding 8-aza-neplanocin A analog displayed some modest activity against HSV-1 and HSV-2, but more importantly, it showed activity against two variants of HCMV at concentrations well below the cytotoxicity threshold.[140] The 8-aza-guanine analog was inactive.[140] Substitution at C2 of the purine aglycon of neplanocin A with fluorine or chlorine resulted in compounds that were completely resistant to deamination by ADA. The fluoro analog had a spectrum of antiviral activity similar to that of neplanocin A, and despite being a poorer inhibitor of S-AdoHcy-ase, it had comparable antiviral potency with apparent less cytotoxicity.[141] More striking changes in the aglycon, represented by the bicyclic heterocyclic moiety of the coformycins, were discovered in nature. Adecypenol (Figure 20) can function as a semi-tight binding inhibitor of ADA, although it is somewhat less potent than coformycin and deoxycoformycin (dCF). This compound, however, would be of interest in combination studies with ara-A because of its lower toxicity relative to dCF.[142] The syntheses of the corresponding cyclopentyl nucleoside, along with the carba-2',3'-dideoxy- and carba-2',3'-dideoxydidehydro analogs, were reported recently.[143] The isomeric cyclopentyl analog where the 4'-hydroxymethyl group is below the plane of the ring was also reported.[144]

Attachment of the aglycon moiety of the naturally occurring exocyclic nucleoside, clitocine, to the cyclopentenyl ring of neplanocin A produced an inactive
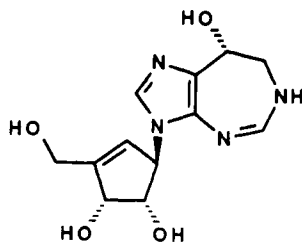

Figure 20. Adecypenol.

compound.[140] This compound was converted to the active 8-aza-neplanocin A mentioned above.[140]

A common problem associated with the use of neplanocin A is the accompanying toxicity that arises from its conversion to the 5'-phosphate.[145] Several approaches designed to circumvent this problem have focused on the synthesis of derivatives that lack substrate properties toward AK, the enzyme responsible for neplanocin's phosphorylation. The first successful approach toward this end came with the synthesis of 3-deazaneplanocin A, which displayed excellent antiviral activity in cell culture against VSV, parainfluenza type 3, yellow fever, and VV. Good antiviral activity was also observed in vivo against VV in a mouse tailpox assay.[146] The significantly lower cytotoxicity of 3-deazaneplanocin A, relative to its parent compound neplanocin A, may indeed be due to its less effective conversion to the 5'-triphosphate metabolite. Modification of the 5'-hydroxymethyl group was also sought as a way of preventing enzymatic phosphorylation and deamination. Consequently, the $X = CH_2OH$ group was changed to $X = -CH_2Cl$, $X = -CH_2SCH_3$, $X = -CH_2F$, $X = -CH_2OCH_3$, $X = -CH=CH_2$, $X = -CH_3$, $X = (R)-CH(OH)CH_3$, and $(S)-CH(OH)CH_3$. All of these compounds were resistant to deamination, but with the exception of the $(5'R)-CH(OH)CH_3$ isomer, none showed appreciable activity against the target enzyme S-AdoHcy-ase (Figure 21).[147] This compound surpassed neplanocin A, both in antiviral potency and selectivity,[147] and was more active than ribavirin against parainfluenza virus (types 2 and 3), mumps, and measles.[148] A recent study also confirmed the anti-VSV activity of the equivalent (5'R)-isomer for the corresponding 3-deaza analog, which additionally was demonstrated to have an excellent selectivity index.[149] A more drastic modification of the molecule consisted in the complete removal of the hydroxymethyl group of neplanocin A to eliminate the possibility of phosphorylation in its entirety. The resulting truncated 9-(trans-2',*trans*-3'-dihydroxycyclopent-4'-enyl)adenine (see Figure 1) and 9-(*trans*-2',*trans*-3'-dihydroxycyclopent-4'-enyl)-3-deazaadenine derivatives indeed showed reduced toxicities, but their antiviral potencies relative to neplanocin A were poorer.[150] These compounds do not inhibit RNA synthesis as neplanocin A, but otherwise they function in an identical manner as inhibitors of S-AdoHcy-ase.[10,151] Neplanocin A, 3-deazaneplanocin A, and the compounds lacking the hydroxymethyl group proved to be specifically effective against VSV, VV, parainfluenza virus, reovirus, and rotavirus in a manner consistent with their effective
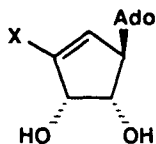

Figure 21.

inhibition of S-AdoHcy-ase. 3-Deazaneplanocin A and the truncated neplanocins had superior selectivity indices when compared to neplanocin A, which could be attributed to their lack of phosphorylation.[152] None of these compounds inhibited HIV replication in MT4 cells.[152] Comparison between the neplanocin A and the aristeromycin analog lacking the hydroxymethyl group showed that the latter was a better inactivator of S-AdoHcy-ase.[53] Of particular interest was the inactivity of the 5'-deoxyneplanocin A in view of the fact that both neplanocin A and the truncated 9-(trans-2',trans-3'-dihydroxycyclopent-4'-enyl)adenine are potent inhibitors of the enzyme.[46] A ring-expanded version of the truncated 9-(trans-2',trans-3'-dihydroxycyclopent-4'-enyl)adenine was weakly inhibitory against S-AdoHcy-ase, whereas the saturated analog was completely inactive.[153]

A comparative study against HCMV between various carbocyclic nucleosides and other noncarbocyclic nucleoside analogs that function as inhibitors of S-Ado-Hcy-ase was performed. The carbocyclic compounds included neplanocin A, 3-deazaneplanocin A, (5'R)-5'-C-methylneplanocin A, 3-deaza-aristeromycin, 5'-nor-aristeromycin, 1'a-β-fluoro-aristeromycin, 9-(trans-2',trans-3'-dihydroxycyclopent-4'-enyl)adenine, 9-(trans-2',trans-3'-dihydroxycyclopent-4'-enyl)-3-deazaadenine, 9-(trans-2',trans-3'-dihydroxycyclopentyl)adenine, 9-(trans-2',trans-3'-dihydroxycyclopentyl)-3-deazaadenine, the two C4' epimers of 5'-deoxy-aristeromycin, and other C4'-modified aristeromycin analogs with 4'β-ethyl, 4'β-vinyl, and 4'β-phenyl subsituents.[136] Neplanocin A and 3-deazaneplanocin A clearly emerged as the more potent compounds, with 3-deazaneplanocin A having the highest selectivity index.[136] All of the C4'-modified derivatives were virtually inactive, including the C4'α-epimer of 5'-deoxy-aristeromycin. As mentioned earlier, although S-AdoHcy-ase is clearly a target for the development of anti-HCMV agents, the anti-HCMV potency for some of these compounds was greater than what would have been predicted on the basis of their inhibitory potency against the enzyme.[136]

In spite of earlier reports regarding the inability of 3-deza-carbocyclic nucleosides to inhibit HIV,[152] some recent investigations indicate that these drugs have therapeutic potential in this area as modulators of important cellular processes.[154] 3-Deaza-aristeromycin and 3-deazaneplanocin A caused a marked reduction in p24 antigen in H9 cells infected with two different strains of HIV-1, and in the chronically infected monocytoid cell lines U1 and THP-1. Similar reductions in p24 antigen were seen in phytohemagglutinin-stimulated peripheral blood mononuclear cells infected with clinical isolates of HIV-1.[154] Surprisingly, the potency of these compounds was greater against AZT-resistant isolates, and although the mechanism of action is not clear, some formation of triphosphate nucleotides was detected, and reduction in syncytium formation in H9 cells was observed.[154]

Additional changes to the carbocyclic moiety of neplanocin A (Figure 22) appear to be detrimental to antiviral activity. For example, the naturally occurring neplanocin C was about tenfold less effective than neplanocin A against VSV.[138] Similarly,
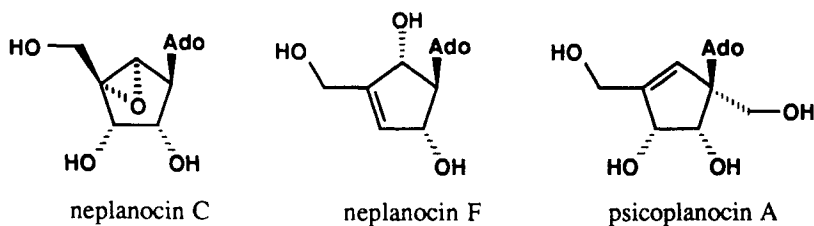
Figure 22. Structures of modified neplanocins.

neplanocin F, the allylic rearranged isomer of neplanocin A, was biologically inactive,[155] and psicoplanocin A, a synthetic hybrid structure between neplanocin A and the ketohexose nucleoside psicofuranin, had only marginal antiviral activity against the arenaviruses Junin and Tacaribe.[156] Conformationally, the extra hydroxymethyl group in psicoplanocin A stabilizes the glycosylic linkage ($\chi = 159.4°$) through the formation of an intramolecular hydrogen bond with the N-3 nitrogen of the adenine ring.[156] The simplest neplanocin A analog, 2',3'-dideoxyneplanocin, was devoid of anti-HIV activity.[157] The presence of fluorine appears to have a definitive effect on ring puckering since the molecule with a 3'-fluoro "up" substituent (xylo configuration) crystallized into two independent structures.[158] One of the structures is conformationally similar to neplanocin A with the fluorine in a pseudoaxial position, whereas the other shows the fluorine in a pseudoequatorial orientation (C2'-*endo*, C3'-*exo*).[158] Although no biological activity was reported for this compound, the cyclopentenyl guanine derivative with a 2'-fluoro "up" substituent (ara-configuration) was a good antiherpetic agent, with a potency equivalent to that of acyclovir.[100]

Pyrimidines

The cytidine analog of neplanocin A, cyclopentenylcytosine (Figure 1), displayed significant antiviral activity against both DNA and RNA viruses. Potent activity was observed for HSV-1 (TK$^+$ as well as TK$^-$), HSV-2, VV, HCMV, and varicella-zoster virus (VZV). Good activity was also reported against a strain of influenza (Hong Kong flu), Japanese encephalitis virus, and Punta Toro virus.[159,160] As the triphosphate, cyclopentenylcytosine blocks the conversion of UTP into CTP and thus shares a similar mechanism of action with the corresponding cyclopentyl analog (carbodine).[161,162] The antiviral activity of cyclopentenylcytosine and carbodine against the TK$^-$ strain of HSV-1 suggests that the common target enzyme, cytidine triphosphate synthetase (CTP-ase), is potentially exploitable for drug design.[163,164] Inhibitors of CTP synthetase such as these also appear to be effective against viruses that cause hemorrhagic fever.[165] A very stringent specificity for both the cytosine aglycon and the cyclopentene ring appears to exist in this case. For example, the 5-azacytosine derivative was ~600 times less potent than the parent

compound in its capacity to reduce CTP levels,[166] and the corresponding 3-deazapyrimidine analogs with 3-deazauracil and 3-deazacytosine bases were devoid of any antiviral activity against HSV-1 and influenza viruses.[167] The setereospecific reduction of the cyclopentene ring of cyclopentenylcytosine resulted in the formation of (–)-carbodine and (–)-isocarbodine. The isomeric (–)-isocarbodine was ~50-fold less potent than (–)-carbodine against human influenza virus.[168] Other cyclopentenyl pyrimidine nucleosides corresponding to 5-amino-imidazole-4-carboxamide riboside, uridine, 5-iodouridine, 4-thiouridine, thymidine, ribofuranosylthymine, 2'-deoxycytidine, 2'-deoxythiouridine, and arabinofuranosyl cytosine were synthesized; however, none showed any effect comparable to cyclopentenylcytosine.[168,170] The corresponding 2',3'-dideoxy analogs of cyclopentenylcytosine[171] and cyclopentenylthymine[75] were devoid of anti-HIV activity in vitro. The latter compound was also inactive against HSV-1 and HSV-2.[75] The rearranged isomers represented by carba-3',4'-didehydrothymidine and carba-3',4'-didehydro-2'-deoxyuridine were also found ineffective against either HSV-1 or HIV viruses in vitro.[172] Finally, the cytosine analog of psicoplanocin A was ineffective as an antiviral agent.[156]

B. Carbovir and Related Nucleosides

carba-2',3'-Didehydro-2',3'-dideoxy nucleosides were first reported as anti-HIV agents in 1988 by Vince et al.[11] Although the guanine base gave the most potent compound with the best selectivity index, other bases with a similar carbocyclic template gave compounds that were classified as active, for example, dideoxyaristeromycin and its didehydro analog. This situation was similar for the corresponding 2,6-diaminopurine analog, whereas both dideoxycarbocyclic and dideoxy-didehydro analogs were also active.[11] However, in the case of guanine, only the dideoxydidehydro analog (carbovir, Figure 1) was active.[11] Replacement of the C8 carbon with nitrogen abolished activity,[173] whereas removal of N-7 gave 7-deaza-carbovir, a compound with a slightly reduced level of potency relative to carbovir against HIV in CEM-SS cells.[174] 7-Deaza-carbovir was also synthesized by another group, who reported on the compound's inactivity against HIV in CEM cells.[175] The data from the two groups agree with respect to the lack of activity of the compound against HSV-1, HSV-2, and HCMV. Carbovir did not appear to have significant activity against influenza A, human rhinoviruses type 14 and type 2, or respiratory syncytial viruses.[176] Recently, 6-deoxycarbovir, a xanthine oxidase-catalyzed activated prodrug of carbovir, was shown to be rapidly converted to carbovir by the enzyme. Since xanthine oxidase activity is present in high amounts in intestine and liver, a high efficiency of conversion is expected to occur in vivo.[177] Intracellularly, carbovir is converted to the triphosphate metabolite and as such it inhibits RT from HIV, AMV, and Moloney murine leukemia virus.[178] Such an inhibition is achieved at concentrations below the K_m for dGTP, but there is a 100-fold variation in the level of sensitivity for the various RTs, with HIV RT being

the most sensitive. On the other hand, carbovir's effect on cellular DNA polymerases α, β, γ and DNA primase appears to be minimal.[178] The stepwise activation of carbovir to the monophosphate level is achieved first by a cytosolic 5′-nucleotidase, which functions preferentially with IMP as the phosphate donor.[179] Indeed, the fact that carbovir triphosphate forms in double-mutant CEM cells lacking dCyK and AK indicates that these enzymes are not responsible for its activation.[179] Of additional interest is carbovir's in vivo transformation to GTP. Although the initial suggestion was that this degradation was caused by the action of purine nucleoside phosphorylase (PNP) during incubation,[179] other studies demonstrated the existence of a chemical degradation process that leads to guanine.[180] The two enantiomers of (±)-carbovir were resolved by the ADA-catalyzed deamination of the racemic 2,6-diaminopurine analog.[181] Inhibition of HIV replication in MT4 cells by carbovir was highly enantioselective, with activity residing only with the (−)-isomer. The first two phosphorylation steps involved in the activation of carbovir to its triphosphate were found to be stereoselective. In contrast, HIV reverse transcriptase was inhibited equally well by both (−)-carbovir triphosphate and (+)-carbovir triphosphate.[181,182] The X-ray structure of (−)-carbovir was described as an envelope conformation with a planar C1′ through C4′ unit and a C1′a-*endo* conformation. This conformation is rarely seen in natural nucleoside analogs and may explain why carbovir is not an adequate substrate for the more common nucleoside kinases. (−)-Carbovir triphosphate probably exerts its antiviral action by a combination of effects, including a direct competition for binding with the natural deoxynucleoside triphosphate and chain termination. Chain elongation studies showed that (−)-carbovir triphosphosphate terminated transcription at positions identical to those where dideoxy-GTP terminated chain growth.[184] The most striking difference between carbovir triphosphate and the other anti-HIV dideoxynucleoside triphosphates (i.e., AZT-TP and ddGTP) is the relative insensitivity of DNA polymerase to carbovir triphosphate.[185] Carbovir has been found to act synergistically with either AZT or ribavirin[186]; however, the cytotoxic effects with AZT were also synergistic.[187] There appears to be no difference between both enantiomers with regard to transport inside the cell,[188] but oxidation of the hydroxymethyl group to the 4′-carboxylic acid derivative[189] is enantioselective, with rat liver cytosol favoring oxidation of the inactive (+)-enantiomer.[190] A glucuronide conjugate was also detected in some species.[191]

3′-Deoxy-2′,3′-didehydro-1′a-*carba*-thymidine, the corresponding thymine analog of carbovir, was inactive against HIV.[192] This is interesting in view of the well-established anti-HIV activity of 3′-deoxy-2′,3′-didehydrothymidine.[193] 2′,3′-Dideoxy-2′,3′-didehydro-1′a-*carba*-7-deaza-adenosine was reported to be tenfold more potent than 7-deaza-carbovir against HIV-1 in CEM-SS cells and slightly less potent than carbovir.[194] Additional 2′,3′-dideoxy-2′,3′-didehydro-1′a-*carba*-nucleosides with the other common and non-common bases have been reported,[195,196] as well as truncated analogs lacking the hydroxymethyl chain; however, their biological activity has not been described.[197,198] Other ring-

expandedversions reminiscent of the structure of carbovir were synthesized, but none were active against HIV.[199]

VI. CARBA-OXETANOCINS

A. Purines

Oxetanocin A, (9-(2R,3R,4S)-3,4-bis(hydroxymethyl)-2-oxetanyl)adenine, is the first and only known example of a naturally occurring four-membered ring nucleoside.[200,201] The reported good antiviral activity of oxetanocin A against HIV sparked a vigorous effort to synthesize and evaluate analogs of this lead compound.[202] The same rationale that suggested the synthesis of carbocyclic furanosyl nucleosides prompted the synthesis of the corresponding carba-oxetanocins, or cyclobutyl nucleosides, to overcome the instability of the oxetanosyl-N-glycosyl linkage. In 1989, Honjo et al. reported the first synthesis of carba-oxetanocin A (the trans-trans isomer, Figure 23) along with the C1'-anomer (cis-trans-isomer).[203] The synthetic guanosine analog carba-oxetanocin G (see Figure 1) became available shortly thereafter, and it too was reported to have excellent antiviral activity. Both carba-oxetanocin A and carba-oxetanocin G at 50–100 μM concentrations provided significant protection to ATH8 cells against the cytophatic effect of HIV-1.[13,204] The anti-HIV activity of carba-oxetanocin G was comparable to that of AZT, but the dideoxynucleosides ddA and ddG exerted their anti-HIV activity over a greater range of nontoxic concentrations.[204] Other purine-modified carba-oxetanocins with bases such as 2,6-diaminopurine, N6-methylaminopurine, 8-bromoguanine, and hypoxanthine were ineffective or weakly protective.[204] Additionally, carba-oxetanocin G had excellent activity, better than acyclovir's, against the herpes virus group (HSV-1 and HSV-2), including HCMV and murine CMV.[205,206] carba-Oxetanocin A, on the other hand, was more potent than acyclovir against VZV, but less potent against HSV-1 and HSV-2.[13] The activity of these carba-oxetanocin nucleosides is also manifested against resistant strains of HCMV, HSV-1, HSV-2, VZV, and HIV-1.[207] The selectivity of carba-oxetanocin G appears to be due primarily to its dependence on virally induced TK. On the other hand, carba-oxetanocin A is equally effective against TK⁻ and TK⁺ strains, but the potency against the latter is reduced by 100-fold.[208,209] Racemic carba-oxetanocin
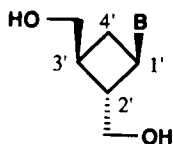
Figure 23. General structure of carba-oxetanocins.

G is thus readily phosphorylated by viral TK functioning as a better substrate for the enzyme than acyclovir. As the triphosphate, it inhibits viral DNA polymerase by competing with dGTP.[210] Metabolically, there is a significant difference between oxetanocin G and its *carba*-isostere. Whereas oxetanocin G exhibits equal potency against the TK+ and TK– strains of HSV-2, the *carba*-isostere experiences a 35-fold reduction in potency.[205] This means that changing from an oxetan ring to a cyclobutyl ring causes a reduction in affinity for cellular kinases, making *carba*-oxetanocin G a more selective substrate for the viral kinase.[205] Therefore, given in combination, *carba*-oxetanocin G and acyclovir compete for viral TK from HSV-1 and HSV-2, whereas oxetanocin G and acyclovir act synergistically against these viruses.[211] The enantio and diastereoselective synthesis of *carba*-oxetanocins A and G demonstrated that the compounds with the configuration that mimics that of the natural nucleosides were highly active, whereas the isomers with the opposite configuration were devoid of activity.[212,213] Conformationally, only in the (–)-*carba*-enantiomers having the 1′R-configuration does the hydroxyl group at C2′ correspond spatially to the 3′-hydroxyl group of the natural nucleosides.[213] (–)-*carba*-Oxetanocin A was very effective against HIV and EBV, whereas (–)-*carba*-oxetanocin G was ~2–3 times more potent than acyclovir as an antiherpetic agent and was comparable to gancoclovir against HCMV.[214] The enzymatically prepared triphosphates of both enantiomeric forms of *carba*-oxetanocin G demonstrated that only the (–)-*carba*-oxetanocin-GTP inhibits HSV-1 DNA polymerase by competing with dGTP, whereas the triphosphate of the (+)-enantiomer was less inhibitory.[213] As opposed to (–)-oxetanocin-GTP, (–)-*carba*-oxetanocin-GTP strongly inhibited DNA polymerase α from calf thymus. Mechanistically, in the case of (–)-oxetanocin G, the termination bands appear mainly at sites two nucleotides beyond the cytosine bases on the template, whereas for the carbocyclic analog, the corresponding termination bands appear most frequently at sites one nucleotide beyond the cytosine base.[215] This situation appears to be similar for HIV reverse transcriptase.[215] Molecular mechanics calculation (MM2) performed on cyclobutyl A and cyclobutyl G indicated that with an energy penalty of 3 kcal/mol the conformations of these molecules show, respectively, a good fit for the conformation of 2′-deoxyadenosine and 2′-deoxyguanosine present in B-DNA. Models of two deoxypentanucleotides were studied, and it was demonstrated that both *carba*-oxetanocin A and *carba*-oxetanocin G can be easily accommodated in double helical polynucleotides with minimal overall distortions.[216] Regarding the first and critical phosphorylation step, it was realized that the rate of phosphorylation of both *carba*-oxetanocin G enantiomers did not correlate with the antiherpetic activity.[217] Surprisingly, it was the inactive enantiomer that was preferentially phosphorylated by HSV-1 TK approximately 20–25 times more effectively than the active enantiomer.[218] With each enantiomer, phosphorylation occurs at the 3′-hydroxymethyl group, which is spatially equivalent to the 5′-hydroxyl group of 2′-deoxyguanosine. However, despite the more effective phosphorylation of (+)-*carba*-oxetanocin G, the ultimate triphosphate metabolite is a poor inhibitor of the viral polymerase,
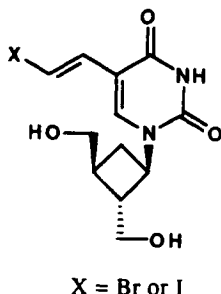
X = Br or I

Figure 24. Carba-oxetanosyl 5-halo-vinyluracil analogs.

whereas the triphosphate of the active enantiomer is an excellent inhibitor of the enzyme. Once (–)-*carba*-oxetanocin-GMP is formed, it is efficiently phosphorylated to the triphosphate, where its long half-life ($t_{1/2}$ = 10 hours) facilitates its accumulation.[218] Following its incorporation into DNA, (–)-*carba*-oxetanocin G is a poor primer for further elongation.[218,219] 2′-Deoxyguanosine (100-fold excess) partially reversed the anti-HSV-2 activity of (–)-*carba*-oxetanocin G,[205] while it abrogated completely its anti-HIV activity.[204] Fluorine substitution in *carba*-oxetanocin G revealed that only the 4′-α isomer was active, although somewhat less potent than the parent compound.[220] Although the active fluorinated (–)-enantiomer was later found to be equipotent to acyclovir, the compound showed significant cellular toxicity.[221] Finally, the aglycon-modified (–)-*carba*-7-deaza-oxetanocin G was synthesized, but no biological activity was disclosed.[222]

B. Pyrimidines

   carba-Oxetanosyl cytosine, uracil, and thymine were ineffective in protecting ATH8 cells against the cytophatic effects of HIV in vitro.[204] The corresponding (±)-*carba*-oxetanosyl 5-(halovinyl)uracil analogs (Cl, Br, and I; Figure 24), on the other hand, had excellent activities against VZV (about tenfold more potent than acyclovir), whereas *carba*-oxetanosyl 5-iodouracil was only weakly active. The compounds were excellent substrates for VZV thymidine kinase and hence were less effective against the TK⁻ strain. The active (1′R) enantiomer of the bromovinyl carbocycle with the "natural" configuration was a poorer substrate for VZV kinase relative to the racemate. This is similar to the case discussed earlier for *carba*-oxetanocin G, where the limited phosphorylation of the active enantiomer appears to be compensated by its rapid conversion to the corresponding di- and triphosphate.[223] Finally, optically pure (–)-4′-α-fluoro-*carba*-oxetanosyl 5-(halovinyl)uracil analogs (Br and I) and 4′-α-fluoro-*carba*-oxetanosyl cytosine were significantly less effective in cell culture against HSV-1, HSV-2, VZV, and HCMV than (–)-4′-α-fluoro-*carba*-oxetanocin G.[221]

VII. MODIFIED *CARBA*-OXETANOCINS

This group includes all *carba*-oxetanocins with a modified cyclobutyl moiety. Inspired by the potent antiviral activity of *carba*-oxetanocin G (Figure 25a) and with the aid of molecular modeling, which suggested that the cyclobutane ring might serve as a surrogate for the tetrahydrofuran ring of the natural 2'-deoxynucleosides, 2'-nor-*carba*-oxetanocin G (Figure 25b) was designed.[224] The compound showed antiviral activity comparable to that of acyclovir against HSV-1, HSV-2, and VZV and was about tenfold more potent than acyclovir against HCMV.[225] The same template with the 5-(2-bromovinyl)uracil aglycon, however, failed to produce an active compound against the same types of viruses.[223] An isomeric template to 2'-nor-*carba*-oxetanocin G and A, where the hydroxyl group has been moved to the 3'-α-position (Figure 25c), gave compounds that were poorly active against
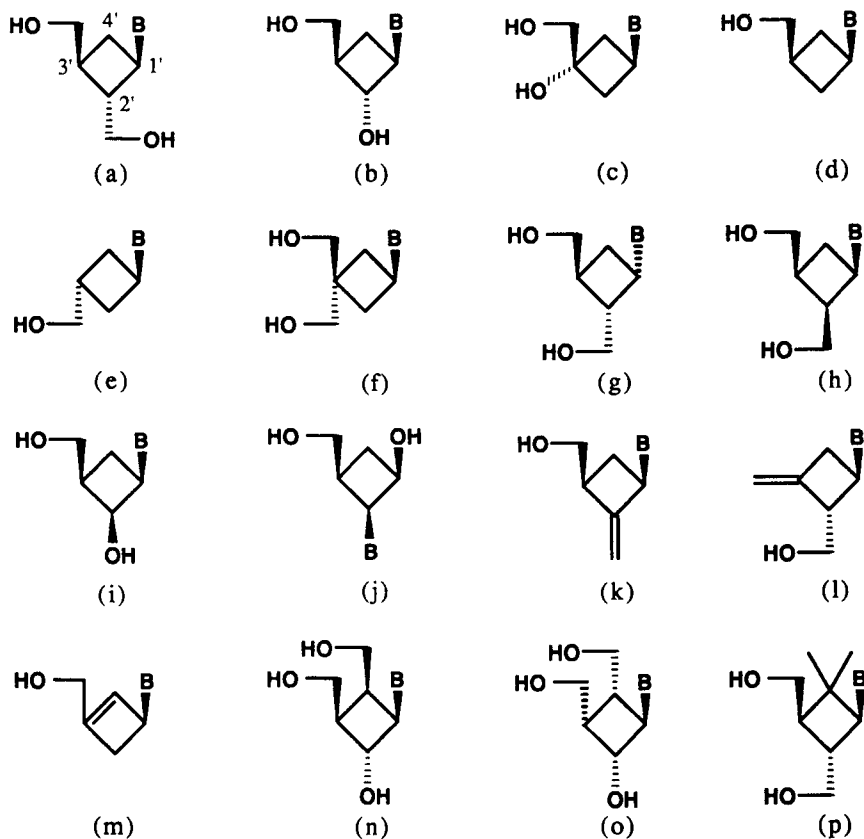
Figure 25. Structural templates of modified *carba*-oxetanocins (B = aglycon).

HIV-1.[226] Also, *carba*-oxetanocin A and G analogs missing one hydroxymethyl group (Figure 25d,e) were inactive against HSV, as were the plain cyclobutyl analogs.[227] Relative to the compounds missing the C2′ hydroxymethyl group, both *cis* and *trans* isomers (Figure 25d,e) were inactive.[227] The 3′,3′-bis(hydroxymethyl) analogs (Figure 25f) were also inactive,[228,229] as was the C1′ anomer of carbocyclic oxetanocin A described earlier (Figure 25g).[203] Surprisingly, the presence of the C2′ hydroxymethyl group does not appear to be essential for good antiretroviral activity, since both *cis* isomers (Figure 25d) with adenine and guanine bases protected cells against the cytophatic effects of HIV in MT2 and ATH8 cells.[227] This means that for anti-HSV activity the presence of both hydroxymethyl groups at C2′ and C3′ and the 1′,2′-*trans* configuration of the purine base relative to the hydroxymethyl group are necessary for activity. On the other hand, the presence of a single hydroxymethyl group at C3′ and the 1′,3′-*cis* juxtaposition of a purine base and hydroxymethyl group are minimally required for anti-HIV activity. The role of the extra C2′ hydroxymethyl group in the latter case would either maintain or increase potency.[227] Since the C2′-hydroxymethyl group was not essential for good antiretroviral activity, the corresponding 7-deaza-adenine and 7-deaza-guanine *cis* isomers were also investigated. However, these compounds lacked anti-HIV activity in CEM cells.[230] Further confirmation that the position of the hydroxymethyl group was critical was provided by a series of 3′,3′-bis(hydroxymethyl) analogs (Figure 25f) with adenine, guanine, 7-deazaguanine, uracil, cytosine, and thymine bases, all of which proved inactive against HSV-1, HCMV, and HIV-1 in cell culture.[229,231] A by-product of the highly stereoselective synthesis of *carba*-oxetanocin A, the isomer where the C2′-hydroxymethyl group is inverted, was obtained (Figure 25h); however, no biological activity was reported.[232] Similarly, the all-*cis* analog 1-[*cis*-2-hydroxy-*cis*-3-(hydroxymethyl)cyclobutyl]thymine (Figure 25i), which is structurally related to 2′-nor-*carba*-oxetanocin G (Figure 25b), was synthesized along with the isomeric 1-[*cis*-2-hydroxy-*cis*-4-(hydroxymethyl)-cyclobutyl]thymine (Figure 25j). Both classes of compounds were ineffective in vitro against HIV-1.[233] Some new *carba*-oxetanocin A and G analogs, in which the cyclobutyl template contains an exocyclic double bond at positions C2′ and C3′ (Figure 25k,l), were disappointing in their biological activity, and only 9-(*cis*-3-hydroxymethyl-2-methylenecyclobutyl)adenine showed marginal activity against HSV-1.[208,209] Additionally, the presence of a double bond in the cyclobutyl ring akin to that found in neplanocin A was investigated for the adenine analog lacking the C2′-hydroxymethyl group (Figure 25m). Its lack of activity is in sharp contrast to the parent cyclobutyl analog (Figure 25d), which was anti-HIV active.[208,234] When the C3′-epimers of 2′-nor-*carba*-oxetanocin G (Figure 25b) were functionalized with an additional β-hydroxymethyl group at C4′, the resulting trihydroxylated *carba*-oxetanocin A analogs (Figure 25n,o) were found inactive against HIV-1 in CEM 4 cells.[235] The incompatibility of extra appendages on the cyclobutyl ring moiety with antiviral activity was confirmed further with the

syntheses of 4,4-dimethyl-*carba*-oxetanocins A and T (Figure 25p), which were also devoid of anti-HIV activity. [236]

VIII. RING-CONTRACTED *CARBA*-OXETANOCINS

Cyclopropylmethyl analogs that were designed as conformationally rigid rotamers of the *carba*-analogs of acyclovir and ganciclovir (DHPG) were synthesized before the *carba*-oxetanocins were known.[237] Both *cis*- and *trans*-hydroxymethyl isomers (Figure 26a) behaved as reasonably good substrates for the HSV-1 TK in comparison with acyclovir, DHPG, and their *carba*-analogs; however, only (±)-9-[[(Z)-2-(hydroxymethyl)cyclopropyl]methyl]guanine, the *trans*-isomer, was active against HSV-1 and HSV-2. Surprisingly, the inactive *cis*-isomer was converted more efficiently to the triphosphate, which was a poor inhibitor of HSV-1 DNA polymerase. Changing the base moiety to 8-azaguanine and 7-deazaguanine modified the substrate's affinity toward HSV-1 TK.[237] The former analog was a competent substrate, whereas the latter was not. Likewise, the thymidine analog was a poor substrate for the kinase, and the bis(hydroxymethyl) analog of *carba*-DHPG (Figure 26a, $R_1 = R_2 = CH_2OH$) was not phosphorylated at all. Although (±)-9-[[(Z)-2-(hydroxymethyl)cyclopropyl]methyl]guanine (Figure 26a, $R_1 = CH_2OH$, $R_2 = H$) displayed good in vitro activity, it failed to prevent or delay death in a HSV-1 murine encephalitis model.[237]

The success of *carba*-oxetanocin A prompted the synthesis of numerous lower methylene homologues of this important broad-spectrum antiviral agent. Among these, the compound having the two hydroxymethyl groups in a *trans* relationship to each other, which corresponds to the true lower homolog of *carba*-oxetanocin A (Figure 26b), became an obvious target. The alternate *cis* isomer (Figure 26c), when analyzed three-dimensionally, appears to have the aglycon's nitrogen and the two hydroxymethyl oxygens in nearly the same spatial relationship as the anti-HIV active 2'-epi-2'-nor-oxetanocin A.[238] Despite these structural analogies, both *cis* and *trans* isomers with adenine and thymine bases proved inactive against HSV-1. The *cis* isomers, however, appear to have some activity against bovine leukemia virus.[239] In the above structures, extending the point of attachment of the base by a methylene spacer (cyclopropylmethyl nucleosides, Figure 26d,e) and maintaining the same relative stereochemistries produced similarly inactive compounds against HSV-1 with adenine, thymine, and 5-fluorouracil bases.[240] The lower homolog of *carba*-oxetanocin G (Figure 26b) was disappointingly inactive against HSV-1 and HSV-2 in Vero cells and was also shown to be inactive against HIV-1 in MT-1 and ATH8 cells.[241] Almost simultaneously, three independent laboratories synthesized a number of racemic 2,2-bis(hydroxymethyl)cyclopropyl nucleosides (Figure 26f).[242-244] The guanine analog was devoid of antiviral activity against HSV-1, HSV-2, VZV, and CMV[242]; the adenine and uracil analogs were reported, but no biological activity was disclosed[243]; and third, a more comprehensive study that encompassed both pyrimidine (thymine, 5-halo (Cl, Br, I)-uracil, 5-bromovinylu-

R$_1$, B   (a)
R$_2$

R$_1$=CH$_2$OH, R$_2$=H (cis)
R$_1$=H, R$_2$=CH$_2$OH (trans)
R$_1$=R$_2$=CH$_2$OH

(b)

(c)

(d)

(e)
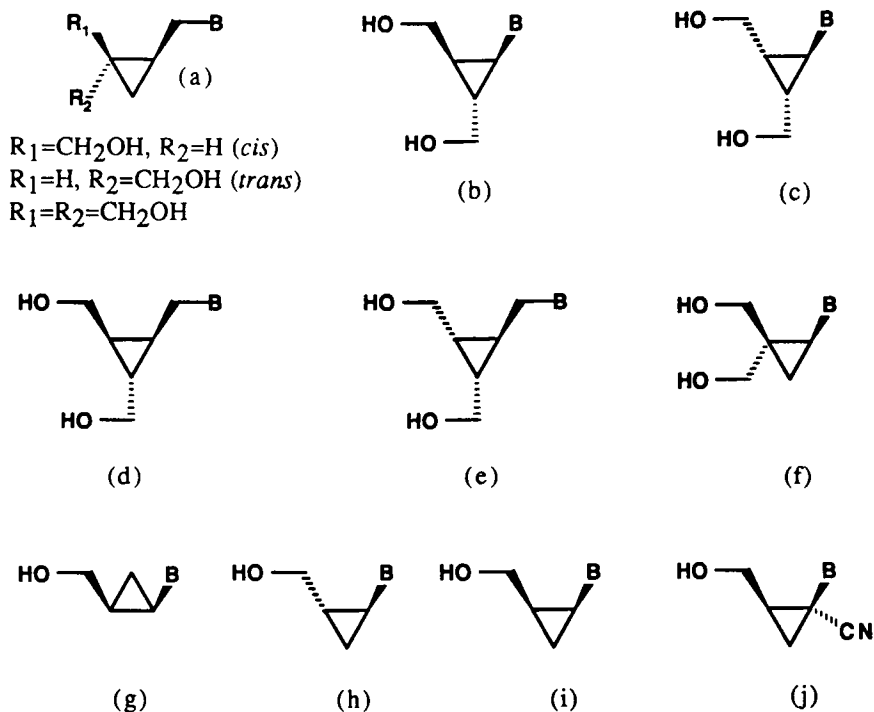
(f)

(g)   (h)   (i)   (j)

Figure 26. Modified ring-contracted *carba*-oxetanocin templates (B = aglycon).

racil, and cytosine) and purine bases (adenine and guanine) reported that none of the compounds were active against HSV-1, HSV-2, HCMV, and HIV-1 in cell culture.[244] Since the presence of the C2' hydroxymethyl group was not essential for the good antiretroviral activity of *carba*-oxetanocin A, the corresponding lower *cis* homolog was synthesized in pure enantiomeric form (Figure 26g) along with the racemic *trans*-homolog (Figure 26h). Although no biological data were provided, the authors asserted that significant biological activity was detected for these compounds and their respective triphosphates.[245] The corresponding enantiomerically pure thymidine analog (Figure 26i) was also reported and found to be inactive against HIV-1 up to 100 μM in human peripheral blood mononuclear cells.[246] Finally, a short synthesis of racemic 1-cyano-2-(hydroxymethyl)cyclopropyl nucleosides (Figure 26j) with uracil, thymine, and cytosine bases was reported, but no biological activity was disclosed.[247]
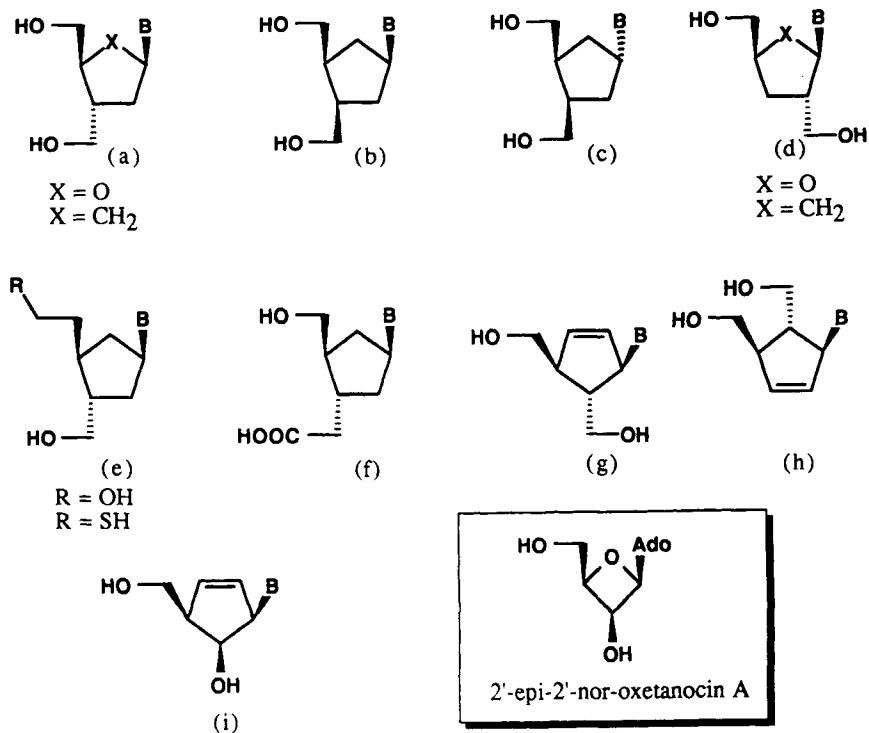
Figure 27. Ring-expanded *carba*-oxetanocin templates (B = aglycon).

IX. RING-EXPANDED *CARBA*-OXETANOCINS

Since 2′,3′-dideoxy-3′C-hydroxymethyl nucleosides (Figure 27a, X = O) were shown earlier to have promising antiviral activity,[248–251] the corresponding carbocyclic analogs (Figure 27a, X = CH₂) became obvious targets. These compounds, which can also be viewed as ring-enlarged analogs of *carba*-oxetanocins A and G, were first reported by Legraverend et al.,[252] and later by Marquez and Buenger, who also prepared the uracil and cytosine analogs.[253] All these compounds were inactive against HIV in either CEM or ATH8 cells.[252,253] Later, the syntheses of the adenine, hypoxanthine, and guanine analogs were described again and reported to have a similar dearth of anti-HIV activity.[254] Analogous compounds, but with two β-oriented hydroxymethyl groups (at C3′ and C4′), and the corresponding regioisomers where the base is below the plane of the ring, were synthesized with the five common bases (Figure 27b,c).[255] None of these, as well as other structurally related pyrimidine nucleosides,[256] were active against a broad range of DNA and RNA viruses and HIV. The isomeric 2′,3′-dideoxy-2′-C-hydroxymethyl carbocy-

clic nucleosides (Figure 27d, X = CH_2) with adenine and guanine bases were synthesized and found to be inactive against HIV.[257] For this class of isomers, the structurally equivalent adenine nucleoside (Figure 27d, X = O) was also reported to be ineffective against HIV.[250] Other ring-expanded versions of carba-oxetano-cins bearing a 4-(hydroxyethyl) or 4-(thioethyl) functionality (Figure 27e) were prepared and used to construct oligonucleotides where a dimethylene sulfone unit replaces the phosphodiester link. No biological activity was reported for the individual monomers.[258,259] Likewise, compounds with a 3'-carboxymethyl substituent (Figure 27f) were synthesized with the intent of incorporating them into nucleic acid templates by simple transesterification. Both adenylic acid and thymidylic acid analogs were constructed, but the individual monomers were not examined for their biological activity.[260] Recently, Katagiri and Kaneko have synthesized an interesting ring-expanded carbocyclic nucleoside that can be construed as a hybrid structure between the anti-HIV active compound carbovir and the carba-oxetanocins (Figure 27g,h).[16] The corresponding adenine and guanine analogs were synthesized first as racemates, but only the adenine analog protected MT4 cells against the cytopathic effects of HIV.[16] Later, the active chiral (1R,4S,6R)-9-(4,6-bis-hydroxymethylcyclopent-2-en-1-yl)-9H-adenine ((–)-BCA, Figure 27g) was resolved and synthetically prepared.[261–263] From the standpoint of structure–activity, this compound can be regarded as a homomethylene derivative of carba-oxetanocin where an ethylene unit is considered to mimic the oxygen atom of the oxetanose ring of oxetanocin A. Resolution of (±)-BCA was also achieved through the action of ADA under high pressure to give exclusively the (–)-hypoxanthine derivative, which was then reconverted to (–)-BCA.[264] Finally, since 2'-epi-2'-nor-oxetanocin A was anti-HIV active,[238] the corresponding (±)-epi-nor-BCA (Figure 27i) was synthesized, but its biological activity was not reported.[265]

X. CARBA-NUCLEOSIDES MODIFIED WITH A HETEROATOM

A novel type of 2',3'-dideoxy-carba-nucleoside template resulting from the transposition of the 4' oxygen to the 3' position (dideoxy-1'a-carba-3'-oxa-nucleosides, Figure 28a) maintains an isomeric relationship with the prototype dideoxynucleosides, but the exchange of oxygen and carbon provides a form of ring puckering that resembles that found in conventional nucleosides. In addition, the structure is still endowed with a stable C-N bond that is typical of carba-nucleosides. Indeed, the first reported member of this series, (+)-iso-ddA, was completely stable under acidic conditions that would normally destroy ddA. In addition, the compound displayed anti-HIV activity in the same range of concentrations as ddA.[17] This compound was also resistant to the action of ADA and appears to be anabolized directly by a nucleoside kinase rather than by way of the inosine derivative, which is the route of activation of ddA.[266] The triphosphate (+)-Iso-ddATP competitively

(a) X=O [(+)-iso-ddB]
(b) X=S
(c) X=N-R (H, OH, CN, etc.)

(d)　　　　(e)　　　　(f)

X=F (h)
X=N3 (i)

(g)　　(-)-iso-ddB (j)　　(k)
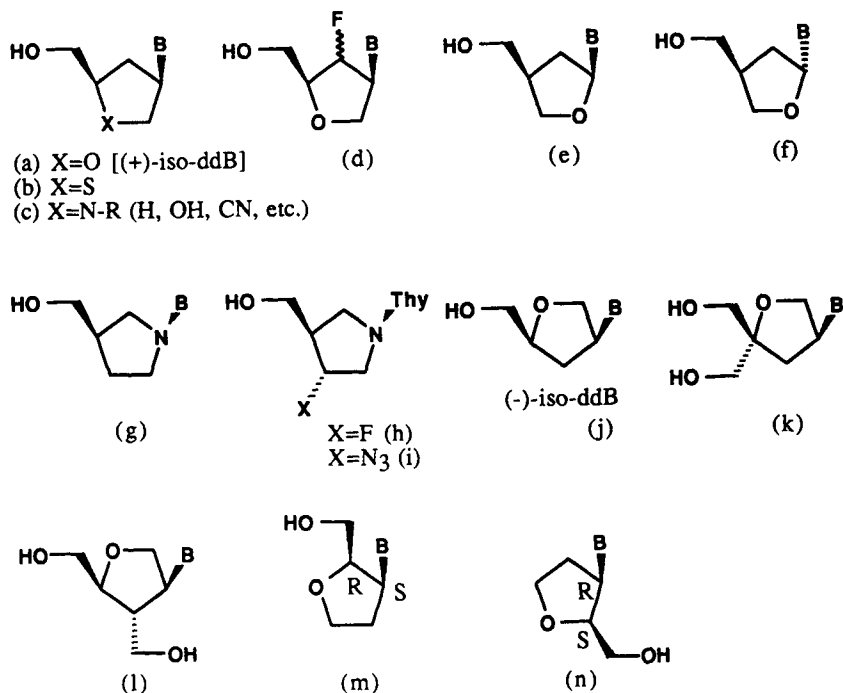
(l)　　(m)　　(n)

Figure 28. Modified *carba*-hetero templates (B = aglycon).

inhibited incorporation of dATP by reverse transcriptase with a K_i ~20-fold higher than that of ddATP,[266] but DNA polymerase α was much more resistant to inhibition. Even though accumulation of the triphosphate anabolite occurs approximately one-third to one-fourth as readily as ddATP, the greater chemical and biochemical stability of (+)-iso-ddA may prove to be advantageous in vivo.[266] The same template was investigated with other bases; however, with the exception of (+)-iso-ddG, which was less potent than ddG, none were active.[267,268] With the same idea, other *carba*-3′-oxa- and *carba*-3′-thia-nucleosides (Figure 28b) with the common bases (uracil, cytosine, thymine, guanosine, and adenine) were investigated. An additional consideration in the design of these compounds was that the electronic environment of the 5′-hydroxyl group was identical to that found in conventional nucleosides, and this was expected to be advantageous in overcoming problems associated with the poor phosphorylation of *carba*-nucleosides. However, as noted earlier, anti-HIV activity was observed only for the *carba*-3′-oxa-nucleosides iso-ddA and iso-ddG.[269,270] These compounds can also be viewed as ring-closed analogs of the acyclic *carba*-derivative, (R)-9-(3,4-dihydroxybutyl)guanine (buciclovir; Figure 29), which is active against HSV-1 and HSV-2 both in vitro and in
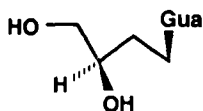
Figure 29. Bucyclovir.

vivo.[271] For this reason, iso-ddG and the 7-deazaguanosine analog were evaluated as anti-herpetic agents. Biological evaluation of these compounds was performed with the chiral 3′-oxa analogs, including the 2-amino-6-chloropurine precursors, but only marginal activity was detected for iso-ddG against HSV-1 and HSV-2.[272] For iso-ddA, changes in the heterocyclic aglycon were not well tolerated. For example, some aza and deaza analogs, including 8-aza, 8-aza-1-deaza, 8-aza-3-deaza, 1-deaza, and 3-deaza-purines, were all inactive against HIV in vitro.[273] The 5′-(phenylmethoxy)alananyl phosphate prodrug of 8-aza-iso-ddA was found active against HIV-1 and HIV-2 with a potency similar to that of iso-ddA, whereas the 5′-(phenylmethoxy)alananyl phosphate of iso-ddA was a better anti-HIV agent than iso-ddA, particularly against HIV-2, where it appears to be as active as AZT.[273] Interestingly, iso-ddATP proved to be a weaker inhibitor of reverse transcriptase than 8-aza-iso-ddATP, even though the former was a much more potent inhibitor of HIV replication in infected cells.[273] The 1′a-α- and 1′a-β-fluoro derivatives (Figure 28d) of (+)-iso-ddA were also synthesized, but biological activity was not reported.[274]

The corresponding dideoxy-*carba*-2′-oxa template (Figure 28e) was investigated, although in this case there is no obvious advantage from the point of view of stability, since an unstable isosteric glycosylic linkage still remains. Racemic prototypes, isomeric to ddU, ddC, and ddG were reported, together with the corresponding α-anomers (Figure 28f), but none of these dideoxy-*carba*-2′-oxa-nucleosides were inhibitory to HIV-1 in vitro at concentrations up to 100 μM.[275] Identical chiral adenosine analogs, which can be considered as derived from dideoxyapiose, were synthesized. In this report, it was claimed that the *cis* (1′S, 4′R) isomer (Figure 28e) had anti-HIV activity in MT-4 cells.[276] Despite having a true glycosylic bond, dideoxy-carba-2′-oxa-nucleosides seem to be slightly more stable to acid cleavage than the corresponding dideoxynucleoside isomers.[277] The corresponding *trans* isomers were also reported.[278]

Comparable nucleosides but with a *carba*-3′-aza template (Figure 28c) having various substituents on the nitrogen (N-X, X = H, CH_2CH_2CN, OH, NO, CN, CO_2CH_3, and CHO) were synthesized with thymine as the base. The compound with X = OH (hydroxylamine derivative) can be considered to be structurally related to thymidine.[279] All of the compounds were inactive against HSV-1 and HIV-1 at concentrations up to 100 μM but displayed significant cytotoxicity against a number of human tumor cell lines.[279] A similar group of compounds, but with

purine bases (adenine, hypoxanthine, 2,6-diaminopurine, and guanine) were found to be inactive against both HIV and HSV-1. In these structures it is important to realize that under physiological conditions the nitrogen of the glycon moiety would be protonated.[280] Moving the nitrogen to the 1′ position resulted in some novel pyrrolidin-1-yl nucleosides. Therefore, a series of 2′,3′-dideoxy-1′a-*carba*-1′-aza-nucleosides (Figure 28g) with uracil, thymine, and cytosine bases were synthesized.[281] Although these compounds could presumably exist as a mixture of α and β forms because of inversion of the pyrrolidinyl nitrogen, NOE experiments confirmed that the anti-β conformation, characteristic of the natural nucleosides, is preponderant in solution.[281] These compounds lacked anti-HIV activity in vitro (in MT-4 cells with HIV-1, RF strain) at concentrations up to 100 μg/ml, where no cell cytotoxicity was even observed.[282] A comparable set of compounds analogous to FddT and AZT (Figure 28h,i) with a similar flexible N-N glycosylic linkage were also synthesized, but they were equally devoid of any useful antiviral activity.[283]

A clever transposition of the adenine base from C1′ to C2′ resulted in a new (−)-iso-ddA analog (Figure 28j, B = Ado), which is the opposite enantiomer of the original (+)-iso-ddA (Figure 28a, X = O, B = Ado) discussed above. Interestingly, (−)-iso-ddA is also a very effective anti-HIV agent. The crystal structures of both (+)-iso-ddA[17] and (−)-iso-ddA[18] are known, and it is interesting to see that the relative disposition of the base and the hydroxymethyl groups is nearly identical in both compounds.[17,18,284] The same transposition as described above was performed with purine and pyrimidine nucleosides having an additional hydroxymethyl group at C4′ (Figure 28k), but no biological data were reported.[285] Both 1′(S)- and 1′(R)-enantiomers were prepared.[285] Conformationally, the former isomers are analogs of (−)-iso-ddA, whereas the latter are analogs of (+)-iso-ddA.[285]

A novel class of branched-chain nucleosides that can be construed as hybrids of ring-expanded oxetanocins and isonucleosides was synthesized.[19] Indeed, transposition of the base from C1′ to C2′ in the active ring-expanded oxetanocin generated this new template (Figure 28l). The adenine, guanosine, cytosine, thymidine, as well as the IDU, BVDU, and IDC analogs were synthesized.[19] The guanine analog (BMS-181,164, Figure 1) displayed potent and selective antiherpetic activity against HSV-1 and HSV-2 that was highly dependent on phosphorylation by viral TK. The compound appeared to be quite selective since it was inactive against VV and HCMV. This compound, although less potent than acyclovir against HSV-1 and HSV-2 in vitro, was quite efficacious in vivo, with ED_{50} values of 84 mg/kg/day (HSV-1) and 52 mg/kg/day (HSV-2) compared to >200 mg/kg/day for acyclovir in the same system.[19] The adenosine analog was not dependent on viral TK for activation and thus it had activity against a wider range of viruses (HSV-1, HSV-2, VZV, HCMV, and VV), but it was somewhat cytotoxic.[19] The cytidine analog likewise was cytotoxic, whereas the thymidine and BVDU analogs showed, respectively, activity against HSV-1 and VZV that was viral TK dependent.[19] The uridine, 5-iodouridine, 5-methylcytidine, and 5-iodocytidine analogs displayed only moderate antiherpetic activity.[19] These new sugar surrogates represent a new template

X=H (AZU-analogue)
X=CH₃ (AZT-analogue)
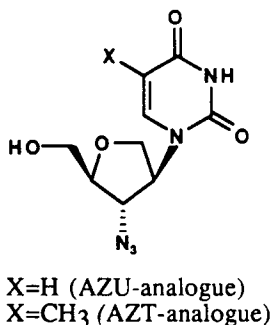
Figure 30.

capable of holding the hydroxyl and nucleobase in a spatial disposition that approximates that found in natural nucleosides. The BVDU analog has shown efficacy against a simian varicella virus preventing the development of rash, decreasing the development of viremia, and preventing death in infected green monkeys treated orally with 4 to 64 mg/kg/day.[286] The same template in which one hydroxymethyl group is replaced by azide, was investigated with thymine and uracil bases, but their biological activity was not reported.[287] These compounds are regioisomers of AZU and AZT (Figure 30) and are structurally derived from 1,4-anhydro-D-ribitol.[287] Other approaches to new isonucleoside templates included isomers of ddA where the adenine base has been transposed to the 3' position (Figure 28m,n). Both optically pure *cis* enantiomers were prepared, but no biological activity was disclosed.[288]

XI. CONFORMATIONALLY CONSTRAINED *CARBA*-NUCLEOSIDES

Some of the ideas related to the importance of conformation in modulating biological activity were discussed in the introduction. Careful consideration of these ideas would suggest that conformationally constrained carbocyclic nucleosides would constitute important tools for studying the importance of this issue. The conundrum, however, is how, in the absence of *gauche* and anomeric effects, a conformation that mimics a typical northern or southern sugar conformation can be imposed on a cyclopentane ring. This problem has been recently addressed. Inspired by the crystal structure of neplanocin C (Figure 22), a naturally occurring carbocyclic nucleoside,[289] the conformationally equivalent bicyclo[3.1.0]hexane system was chosen to generate rigid nucleosides with a conformation typical of a northern hemisphere geometry (2'-*exo*/3'-*endo*).[290,291] The interesting property of these bicyclic systems, be it the 6-oxa-bicyclo[3.1.0]hexane of neplanocin C, or the plain bicyclo[3.1.0]hexane, is that they are rigid and exist exclusively in a
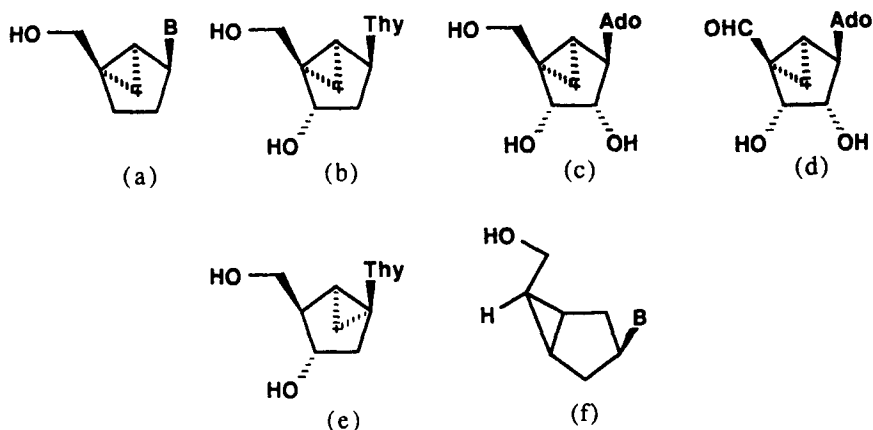
Figure 31. Conformationally constrained *carba*-nucleoside templates (B = aglycon).

pseudoboat conformation. The corresponding dideoxynucleoside series, built on the bicyclo[3.1.0]hexane template (Figure 31a), was synthesized and evaluated for anti-HIV activity as rigid northern conformers. Only the adenine analog showed a significant level of protection that was overcome by cytotoxicity.[291] Analogously, but on the basis of experimental and theoretical studies, the same bicyclo[3.1.0]hex-ane system was selected by another group expecting a conformation closely related to the 2'-*exo*/3'-*endo* conformation of the sugar moieties found in A-type double helices.[292] Although the purpose of this work was to incorporate these modified carbocyclic nucleosides into oligodeoxynucleotides for antisense studies, it re-ported the first synthesis of the carbocyclic analog of thymidine (Figure 31b) built on this new rigid template.[292] Interestingly, this compound was found to be a very potent and selective antiviral agent against RSV.[293] Furthermore, the corresponding *carba*-ribo analog of adenosine (Figure 31c) was synthesized and evaluated to-gether with the 5'-carboxyaldehyde derivative (Figure 31d) for their inhibitory effect on *S*-AdoHcy-ase. However, in contrast to the potent inhibitory activity of both aristeromycin and adenosine-5'-carboxaldehyde,[294] these compounds were weak inhibitors of the enzyme.[293] The isomeric carbocyclic thymidine with the cyclopropane ring moved toward the base (Figure 31e) gave a compound with a form of ring puckering characteristic of the alternate 2'-*endo*/3'-*exo* conformation found in canonical B-DNA duplexes.[295] Although this compound has not been tested for antiviral activity, it represents a novel template that might overcome some of the shortcomings of carbocyclic nucleosides. Both of these rigid templates, substituted with the appropriate bases, might provide some additional insight into the preferred mode of binding of nucleosides with their target enzymes. Some other

$$X=CH_2 \text{ (a)}$$
$$X=CF_2 \text{ (b)}$$
$$X=CH_2O \text{ (c)}$$

9-(5-phosphonopentyl)adenine

(d)

PMEG

(e)

(f)   **(g)**

(h)

X = H (i)
X = F (j)
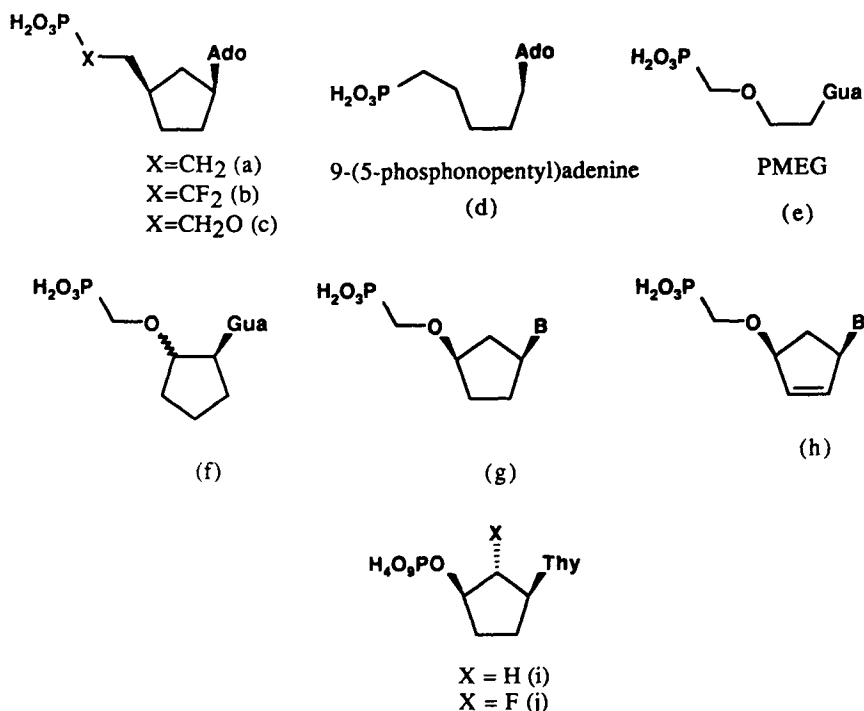
Figure 32. *Carba*-nucleotide phosphonate templates (B = aglycon).

more exotic forms of cyclopropane-fused carbocyclic nucleosides have been re-
ported (Figure 31f), but their biological activity remains unstudied.[296]

XII. *CARBA*-NUCLEOTIDES AND PHOSPHATE MIMICS

Phosphonate groups that function as phosphate mimics have been designed to
attempt to overcome the required first phosphorylation step in nucleoside analogs,
which is usually rate limiting and often proceeds very poorly. Carbocyclic phos-
phonate derivatives of ddAMP in which the oxygen atom connected to the phos-
phorous was replaced by CH_2, CF_2, or CH_2O have been synthesized (Figure
32a,b,c).[297] The first two are phosphonate isosteres with a P-C-C chain linked to
C4', whereas the third has an extended P-C-O-C link connected to C4'. None of
these compounds, however, were phosphorylated by adenylate kinase in the present
of ATP.[298,299] On the other hand, the conventional monophosphate of aristeromycin
(*carba*-AMP), as well as the corresponding 1'a-α-fluoro derivative, were good
substrates for adenylate kinase and pyruvate kinase working in tandem.[300] Even the
corresponding 9-(5-phosphonopentyl)adenine (Figure 32d), which can be regarded

as an acyclic derivative of carbocyclic ddAMP, was more efficiently phosphory-lated than the carbocyclic phosphonates, indicating that the pseudoribose ring was not critical for substrate recognition.[299] The phosphonomethyl analog of ddATP was also synthesized along with the 2′,3′-unsaturated derivative, which was ob-tained as an inseparable mixture with the 1′,2′-isomer.[301] Following the lead provided by the very active antiherpetic agent 9-[2-(phosphonomethoxy)ethyl]gua-nine (PMEG, Figure 32e),[302] several related guanine derivatives, in which the acyclic chain connecting the phosphonomethoxy group and the guanine base was replaced by a cyclopentane ring, were synthesized. Although the 1,2-substituted cyclopentane derivatives (Figure 32f) deviated too much from a nucleotide-like structure, the 1,3-substituted derivatives (Figure 32g) could be oriented in the same relative configuration as the base and the phosphate in naturally occurring nucleo-tides.[303] The cyclopentene derivative (Figure 32h), which represents an isosteric and isoelectronic analog of the monophosphate of carbovir, was also synthesized.[303] However, this compound was inactive as an antiherpetic agent.[303] Against HIV, the *cis*-1,2-substituted cyclopentane isomer showed moderate antiviral activity, whereas the *trans* derivative was inactive.[303] In the 1,3 series, the unsaturated derivative (the carbovir analog) displayed only modest activity, whereas the satu-rated analog was inactive.[303] Chiral resolution of the active 1,3-unsaturated enantiomer indicated that it had the same relative configuration as the active isomer of carbovir.[303] The same phosphonate analog of carbovir just described, plus addi-tional saturated and unsaturated dideoxycarbocyclic nucleoside analogs with ade-nine, hypoxanthine, cytosine, uracil, and thymine bases were also reported, but no biological data were disclosed.[304] Interestingly, the racemic isosteric and isoelec-tronic analog of carbovir triphosphate, technically the diphosphorylphosphonate of 5′-norcarbovir, was a potent inhibitor of HIV-RT.[305] The ensuing preparation of both enantiomers revealed that the unnatural triphosphate mimic (see Figure 1) was more potent than the natural mimic.[15] Since the compound is inactive as an anti-HIV agent, possibly because of a lack of intracellular transport, a prodrug of this triphosphate mimic is being pursued as a less toxic and more specific agent.[15] A similar attempt with 5′-norcarbocyclic thymidine and its 1′a-α-fluoro derivative confirmed that a diphosphorylphosphonate unit indeed is a perfect isostere of a 5′-triphosphate (Figure 32i,j).[306] Despite the fact that the phosphonate analogs were devoid of anti-HIV activity in vitro, the diphosphorylphosphonates, particularly those of 5′-norcabovir and the fluorinated thymidine analog, had spectacular activity against HIV reverse transcriptase ($IC_{50} = 0.06$–0.43 μM) in the same range of potency as carbovir triphosphate, and were somewhat less potent than AZT triphosphate.[306] A three- and four-methylene extension between the oxygen and the phosphonomethoxy group, which was intended to increase the lipophilic nature of the phosphonates, failed to give any active compounds.[305] Surprisingly, the triphos-phates of 1′a-α-fluoro-3′-deoxy-*carba*-thymidine and the corresponding 1′a-α-fluoro-*carba*-AZT displayed a low level of RT inhibitory activity, and the triphosphate of 5′-norcarbocyclic thymidine was found to inhibit the enzyme, albeit

5'-nor-*carba*-BVDU   3'-nor-*carba*-oxetanocin
phosphonomethyl analogue   phosphonomethyl analogue
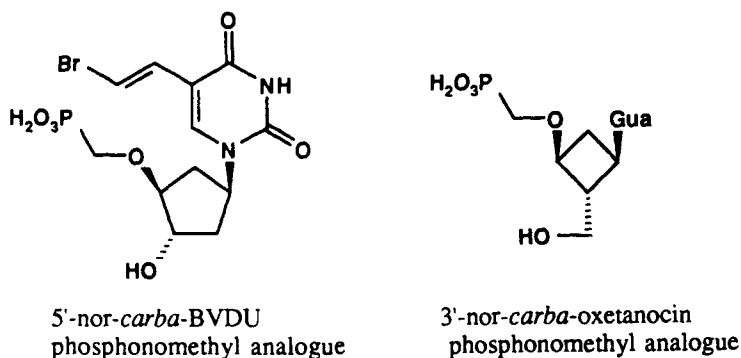
Figure 33.

at a level two orders or magnitude weaker than AZT-TP ($IC_{50} = 7.9$ µmol).[307] This means that the diphosphorylphosphonates of the corresponding 5'-nor-carbocycles are indeed perfect 5'-triphosphate mimics. The equivalent racemic phosphonate isostere of *carba*-BVDU monophosphate, which is in essence a 5'-nor-*carba*-BVDU phosphonate (Figure 33), was completely inactive against HSV-1 in vitro.[308] Other 5'-nor-phosphonate analogs with a P-C-O link to C4' that have been reported include phosphonates of 2'-deoxy-5'-nor-*carba*-guanine and 6-mercaptopurine. However, only in vitro cell cytotoxicity was reported for these compounds.[309] A rationale similar to the one described above led to the synthesis of the phosphonate isostere of the monophosphate of (±)-*carba*-oxetanocin G (Figure 33). This compound is a phosphonomethyl isostere with a P-C-O link constructed by reversing the carbon and oxygen in (±)-*carba*-oxetanocin GMP. Although the compound showed some antiviral activity against HSV-1 and HSV-2, the potency was about two orders of magnitude lower than that of *carba*-oxetanocin G.[310] Activity against HCMV was moderate, whereas against HIV activity was totally lacking.[310]

3-(Phosphonomethoxy)pyrrolidin-1-yl derivatives (Figure 34) of purines and pyrimidines, which correspond structurally to phosphate mimics (phosphonates) of 2',3'-dideoxy-5'-nor-1'a-*carba*-nucleosides with a P-C-O link and a flexible N-N
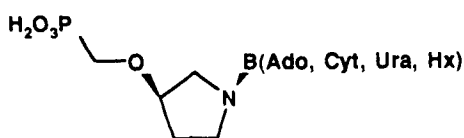
Figure 34. 3-(Phosphonomethoxy)pyrrolidin-1-yl analogs.

carba-c-AMP (a) carba-neplanocin-c-AMP (b) (c)

carba-griseolic acid derivative (d)
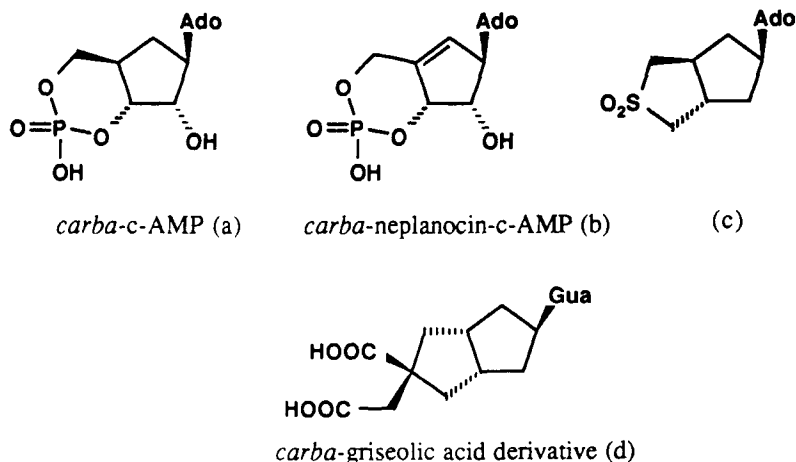
Figure 35. Carba-phosphonate analogs of cyclic nucleotides.

glycosylic linkage, were synthesized.[311] The corresponding analogs of dideoxyuridine, dideoxythymidine, dideoxycytosine, dideoxyadenosine, and dideoxyinosine were virtually inactive against HSV-1 and HSV-2, and only the dideoxyadenosine analog showed good activity against Visna virus in sheep coroid plexus.[311]

Carbocyclic analogs of cyclic nucleotides have also been reported. The carbocyclic analog of cyclic AMP (Figure 35a) and the corresponding neplanocin analog (Figure 35b) were synthesized, but their antiviral activity was not studied.[312] However, the compounds behaved as strong inhibitors of cyclic AMP phosphodiesterase.[312] An uncharged analog of 2'-deoxy-1'a-carba-adenosine-3'5'-monophosphate, where the phosphate diester monoanion was replaced by a dimethylene sulfone unit on a carbocyclic template, was also synthesized (Figure 35c), but no biological activity was reported either.[313] Griseolic acids, which can function as mimics of cyclic AMP and behave as competitive inhibitors of cyclic nucleotide
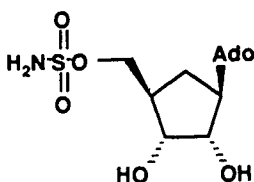
Figure 36. 5'-Sulfamoyl-carba-adenosine.

phosphodiesterases, provided the incentive to synthesize the corresponding *carba*-nucleosides. A series of *carba*-griseolic acid analogs with guanine, hypoxanthine, and 2-amino-6-chloropurine bases was synthesized where the *carba*-sugar portion included cyclopentane as well as cyclopentene rings. The compounds were evaluated as inhibitors of cyclic GMP phosphodiesterase but their antiviral activity was not evaluated.[314] Finally, 5'-sulfamoyl carbocyclic adenosine (Figure 36) and 5'-sulfamoyl-8-aza carbocyclic adenosine were synthesized, but only their cytotoxicity against P388 mouse leukemia cells was reported.[315]

XIII. *CARBA*-NUCLEOTIDE OLIGOMERS

In addition to the carbocyclic bicyclo[3.1.0]hexane nucleosides described above, other more conventional *carba*-nucleosides have been incorporated into oligonucleotides. For example, the incorporation of aristeromycin into a 2-5A (5'-monophosphate) trimer core similar to adenilyl(2' → 5')adenilyl(2' → 5')adenosine, led to a decrease in potency, relative to the parent oligomer, in its capacity to cleave rRNA. The parent trimer is believed to be a mediator of at least some of the antiviral actions of interferon.[316] 2'-Deoxyaristeromycin has been incorporated into oligodeoxynucleotides[317] and a double-stranded d(GGAriAGG) oligomer corresponding to the bleomycin-cleaving site. In this oligomer an unprecedented bleomycin-mediated dehydrogenation occurred effectively at the C4' and C1'a positions of 2'-deoxyaristeromycin (Ari), transforming it into 2'-deoxyneplanocin A in the intact oligo.[318] Carbocyclic oligothydmidylates appear to have increased stability toward enzymatic degradation, which when coupled to their ability to bind more strongly to complementary DNAs, suggests that these *carba*-nucleosides might be of use in the antisense field.[319,320] Short oligonucleotide segments containing 1'a-α-fluoro-1'a-*carba*-thymidine were prepared from the corresponding phosphoramidite. Hybridization between a natural DNA oligomer and a fluorinated nucleotide mimic, as well as hybridization of two complementary fluorine-containing strands, was successful. In these oligomers ^{19}F NMR studies could be useful in detecting conformational changes during hybridization.[321] The carbocyclic analog of 5-methyl-2'-deoxycytidine was found to stabilize triple-helix complexes when incorporated into an oligodeoxynucleotide, relative to the furanosyl nucleoside. This effect is due in part to the increased basicity of the heterocycle that facilitated protonation at physiological pH and stabilized the triple-helix complex.[22] *carba*-Thymidine had the opposite effect, destabilizing the triple-helix complex, possibly because of its unusual C1'-*exo* pucker.[22] Finally, octameric phosphodiesters of 1'-thyminyl- and 1'-adenyl-3',3'-bis(hydroxymethyl)cyclobutane (*carba*-oxetanocin analogs) were prepared to study their potential for annealing and homologous hybridization.[322,323]

XIV. MISCELLANEOUS

A. *carba*-C-Nucleosides

Recently the carbocyclic analog of tiazofurin was reported. The compound was not examined as an antiviral agent and showed one-tenth the cellular cytotoxicity of tiazofurin toward a breast carcinoma cell line.[324] Other *carba*-C-nucleosides reported included *carba*-oxazinomycin[325] and *carba*-C-tetrazole (Figure 37).[326]

B. 5'-Homo-*carba*-Nucleosides

(–)-5'-Homoaristeromycin, with an extended 4'-hydroxyethyl group, was prepared from L-ribonolactone, and it showed disappointing antiviral activity. It is presumed that the primary hydroxyl group is not phosphorylated by cellular or viral kinases.[327]

carba-tiazofurin   *carba*-oxazinomycin   *carba*-C-tetrazole

Figure 37.

(a)   (b)
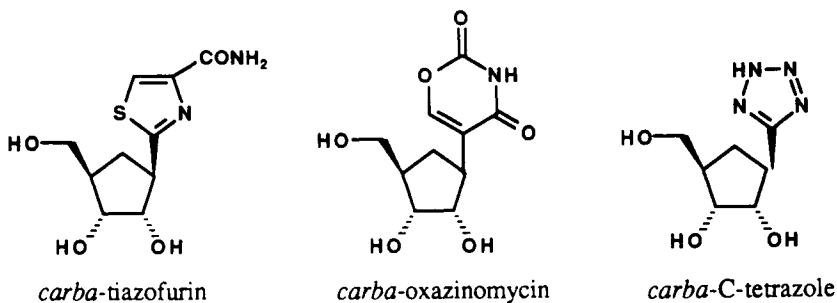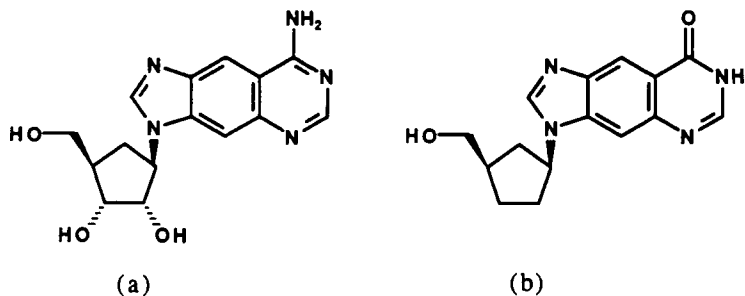
Figure 38.

C. *lin*-Benzo-Separated *carba*-Nucleosides

The syntheses of *lin*-benzo-separated aristeromycin (Figure 38a)[328] and the corresponding 2′,3′-dideoxyinosine analog (Figure 38b)[329] were reported, but no biological activity was disclosed.

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COMMENTS ON NUCLEOTIDE DELIVERY FORMS

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I. INTRODUCTION

A multitude of factors affect the biological activity of nucleoside analogs (Nu) which, in most cases, must be phosphorylated intracellularly by cellular or viral enzymes before exerting their effects. It follows that the intracellular metabolism of nucleosides is a key event for the appearance of their biological response.[1,2]

Dividing, long-term cultured cell lines have been used frequently as target cells for the study of *in vitro* activity of nucleoside analogs. Although this may be appropriate for anticancer nucleosides, it has key limitations for the evaluation of anti-HIV nucleosides whose targets are predominantly quiescent mononuclear cells (monocytes/macrophages, dentritic cells, and glial cells), which may differ in their metabolizing capacities. The amount of nucleoside phosphorylation varies considerably between cell lines.[3,4] Therefore, one must keep this in mind, as the following considerations and discussions will be mainly based on nucleoside metabolism and on their *in vitro* antiviral activities.

In all the nucleoside series with the same sugar modification, the biological responses differ greatly according to the type of nucleobase (as an example compare the dideoxynucleoside activities toward HIV-infected cells). The most probable reason for this phenomenon has to be seen in the ability of the nucleosides to be phosphorylated to the corresponding 5'-triphosphates (NuTP), which depends on the activity and specificity of the viral and/or cellular phosphorylating enzymes.

Among the three successive activating phosphorylation steps the first one is the most crucial.[5] Several different enzymes may perform this first phosphorylation step, depending on the nature of the aglycone.

Nucleosides may be first converted to their corresponding 5'-mononucleotides (NuMP) by cellular or viral enzymes. For example, 3'-azido-3'-deoxythymidine (AZT) and 2',3'-dideoxycytidine (ddC) are monophosphorylated by cellular deoxynucleoside kinases and 9-(2-hydroxyethoxymethyl)guanine (acyclovir) by herpes virus-induced thymidine kinase (TK). Formation of the NuMP may also depend on nucleotidases as for 2',3'-dideoxyinosine (ddI) and C-2',3'-didehydro-2',3'-dideoxyguanosine (carbovir) or on other activating enzymes present naturally in cells.[1,4]

At this point we would like to emphasize the fundamental importance of the initial phosphorylation, which is the rate-limiting step, as further phosphorylations to the di- and triphosphates are accomplished by relatively less specific and less stringently regulated nucleotide kinases. This point is exemplified by the following remarks:

1. Some nucleosides do not show a biological response only because they are not enzymatically transformed to the corresponding NuMP. As an example, 2',3'-dideoxyuridine (ddU) does not present any anti-HIV effect in cell culture, but liposome delivery of ddUMP leads to an antiviral response.[6]

2. The presence and activity of the intracellular enzymes necessary for activation of nucleoside analogs are highly dependent on host species, cell type, and stage in the cell cycle.[4] In this respect, it has been suggested that in human peripheral blood mononuclear cells (PBMC), the activity of uridine kinase is likely to be cell type specific and dependent on cell growth.[7] In addition, AZT preferentially protects activated PBMC against HIV-1 infection as compared to resting cells.[8] This point may be explained by the fact the TK is an S phase-specific enzyme, whose activity is increased by several orders of magnitude during cell division.[4] On the

other hand, ddl exerts antiviral activity more favorably in resting cells,[8] where the nucleotidase activity remains relatively constant during all phases of the cell cycle.[9]

3. It should also be noted that the emergence of resistance to nucleoside analogs has been frequently attributed to a decrease or loss of activity of the first phosphorylating enzyme. For instance, resistance to acyclovir can arise by mutation in the *tk* gene, and three different kinds of mutations have been observed in cell culture giving rise to TK-negative (TK⁻), TK-partial, and TK-altered mutants. As another example, gancyclovir has been shown to be phosphorylated by a protein encoded by the human cytomegalovirus *UL97* gene, which upon mutation can also give rise to apparition of a resistance to this drug.[10] It follows in such cases that overcoming the specific viral enzyme might be counterproductive in terms of selectivity.[11] However, in most cases, nucleosides (i.e., anti-HIV nucleosides) are dependent on cellular kinases for phosphorylation.

For all these reasons, the intracellular NuMP delivery has been considered for overcoming the first phosphorylation step.

Nucleotides themselves cannot be used as potential chemotherapeutic agents. Because of their polar nature, they are not able to cross the cell membrane efficiently. Moreover, they are readily dephosphorylated in extracellular fluids and on cell surfaces by nonspecific phosphohydrolases. Therefore, it has been proposed for many years to use nucleotide prodrugs, modified on the phosphate moiety, with the expectation that the reduced charge would enable the modified nucleotide to enter the cell intact and, once within, be converted to the parent nucleotide (Figure 1).

With regard to such an approach some fundamental questions arise on the stability of the nucleotide derivative under the experimental evaluation conditions, the selectivity of hydrolysis to the expected NuMP, and the toxicity induced by the promoiety of the prodrug.

We would like to emphasize the following comments concerning the stability of the nucleotide prodrug in cell culture media upon incubation conditions (37°C). First, cell culture medium contains in most cases 5–10% of inactivated serum (usually heated at 56°C for 30 min). However, it has been shown that some enzymatic activities (phosphodiesterase, phosphatase, etc.) could be still present in the medium.[12,13] Second, if any decomposition of the prodrug occurs in the culture medium, it will release extracellularly the parent nucleoside, which will be taken up. This implies that if one wants to demonstrate intracellular NuMP delivery on the basis of a biological response, one must conduct the experiments on cell lines deficient in the first nucleoside-activating enzyme (i.e., TK⁻ cell lines for AZT prodrugs). One can also use an inactive Nu, for which the corresponding NuTP is a viral polymerase inhibitor and which has been shown to be a nonsubstrate for the first phosphorylating enzyme.[14]

The NuMP delivery problem has been addressed by numerous laboratories over the course of about 30 years, and we will try to evaluate the biological results related to the various strategies reported to date.
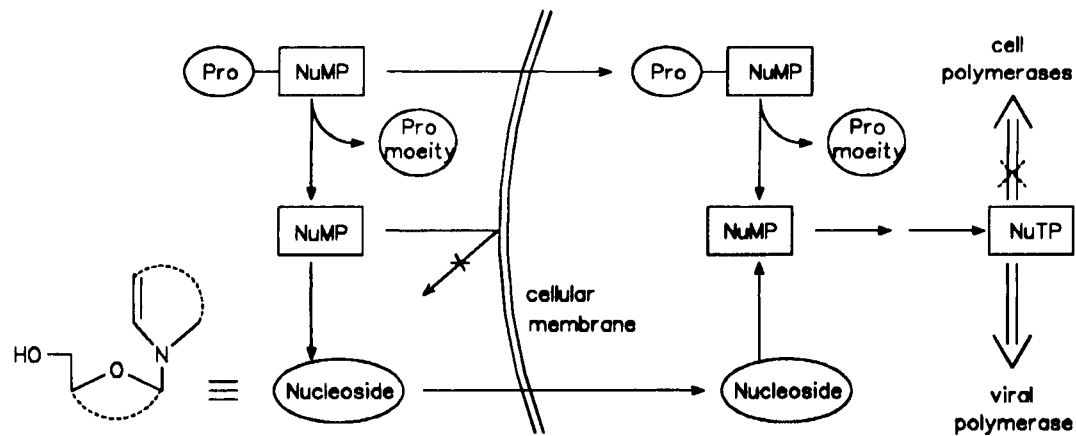
Figure 1.

II. THE PHOSPHODIESTER APPROACH

A phosphodiester prodrug can be designed either from a mononucleoside or a dinucleoside derivative (Figure 2), the latter being characterized by a dinucleosidyl-3′,5′ or -5′,5′ structure, both series being expected to be enzymatically cleaved to the corresponding NuMP.

Some questions on the mechanism of action of such phosphodiester prodrugs may arise as follows:

1. Are such charged phosphodiesters readily taken up? Obviously, the answer to this question is related to the overall lipophilicity of the considered phosphodiester. Some mononucleoside phosphodiesters, in which R is a very lipophilic substituent, have been shown to be taken up, at least partly.[15]

2. Are such specific phosphodiesters substrate for phosphodiesterases and what about the cleavage selectivity? In this respect, it was reported that some phosphodiesters (Figure 2) ware substrates for snake venom phosphodiesterase.[16–18] However, spleen phosphodiesterase only cleaves the 3′,5′-phosphodiester of araC (to give the 3′-NuMP) and not the 5′,5′-isomer.[18] Regarding 6-mercaptopurine, its dinucleosidyl-5′,5′ phosphodiester was shown to be a good substrate for the phosphodiesterases present in cell-free extract of HEp n°2.[16] Moreover, in CEM cell extracts the 5′,5′–phosphodiester of ddC appears to be stable, whereas in culture medium its half-life ($T_{1/2}$) is about 48 h[12] (note that under the same conditions, dinucleosidyl-3′,5′ phosphodiesters are usually about 10 times less stable[10]). These last data suggest that phosphodiesterases present in the extracts may degrade the phosphodiesters, but to various extents depending on their structure. In plasma of various origins such degradation of 5′,5′-isomers arises most readily.[20,21]

3. As such prodrugs are designed for intracellular NuMP delivery, what about the distribution of the hydrolyzing enzymes? Since phosphodiesterases are present in the cell and also in serum, the prodrugs may be at least partly hydrolyzed to the corresponding NuMP and Nu before entering cells. In this regard, the phenylphosphodiesters of 2′,3′-didehydro-2′,3′-dideoxyadenosine (d4A) and of 2′,3′-didehydro-2′,3′-dideoxycytidine (d4C) have been shown to be rapidly hydrolyzed in culture medium (containing serum) with formation of the corresponding NuMP and Nu. Hence, the authors, concluded that such compounds can function as prodrugs of antiviral nucleosides that are released before their introduction into the cells.[13]

Therefore, if one considers a nucleoside phosphodiester, one must carefully consider all of the above remarks before inferring intracellular NuMP release on the basis of biological responses. In fact, as it was previously pointed out, one of the main problems arising with such a prodrug approach is devising an assay system that can show unequivocally whether the principles are sound.[22]

We will now briefly present some of the main phosphorylated nucleoside series that have been proposed by various groups with the aim of intracellular NuMP delivery. Previous works based on overcoming the resistance of antitumor nucleo-

5',5'-Dinucleoside phosphodiester

Mononucleoside phosphodiester

3',5'-Dinucleoside phosphodiester
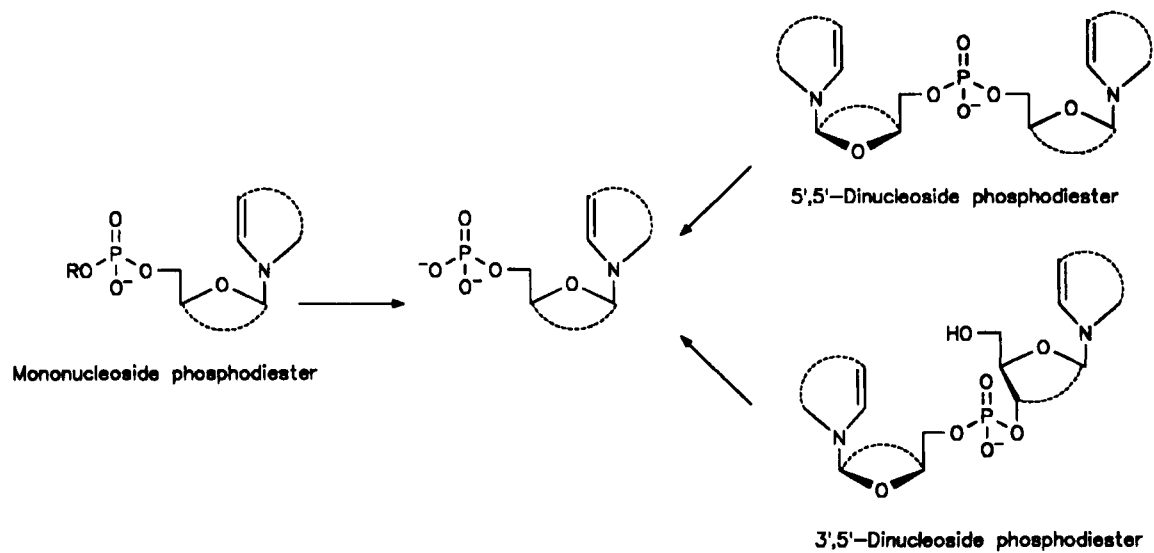
Figure 2.

sides have been reviewed.[23-25] For instance, some lipophilic 5′-alkylphosphate esters of araC showed antileukemic activity in mice comparable to or greater than that of the nucleoside parent. However, activity against a kinase-deficient resistant cell line *in vivo* was not observed.[24]

The prodrug approach using alkyl (or aryl) phosphodiester derivatives has also been applied to antiviral nucleosides. Some 5′-methyl and 5′-phenyl phosphodiesters of d4A and d4C along with two 3′,5′-phosphodiesters involving thymidine were evaluated for their inhibitory effect on the replication of various DNA and RNA viruses.[13] The phosphodiesters of d4A and d4C proved inhibitory only to the cytopathicity of HIV-2 and HIV-1, and at concentrations comparable to those of the parent compounds. As the didehydronucleosides present some instability in solution (cleavage of the glycosidic bond),[26,27] it is noteworthy that the corresponding phosphodiesters showed a variable tendency toward decomposition in D_2O, in relation to their structure.

Upon incubation in culture medium (RPMI 1640 containing 10% of fetal calf serum), the phenylphosphodiester of d4A decomposes after 5 hours to d4AMP (28%) and d4A (9%). These products increased in 23 hours to 38% and 41%, respectively. It was therefore concluded that these compounds can function as prodrugs of their parent nucleosides, which are released before their uptake by the cells.[13]

Another series for phosphodiesters involves steroid conjugates of nucleoside analogs. Conjugates of cortisol, cortisone, corticosterone, prednisolone, and prednisone linked from position 21 of the steroid to the 5′ position of araC *via* a phosphodiester bond have been studied.[24] The efficacy observed was dependent on the nature of the steroid. These compounds were resistant to cytidine deaminase from human liver and to alkaline phosphatase but were sensitive to phosphodiesterase, 5′-nucleotidase, and acid phosphatase. Like the simple alkyl phosphate esters, the conjugates of cortisol and corticosterone showed only marginal activity against a kinase-deficient leukemia resistant to araC. Another steroid conjugating moiety, 7-β-hydroxycholesterol, chosen for its lipophilic character and its actions on the cell membranes,[24] was initially studied with 5-fluoro-2′-deoxyuridine (5F-dUrd) as the nucleosidic model.[24,28] More recently, the synthesis of monophosphoric acid diester of 7-β-hydroxycholesterol and AZT has been reported.[29]

Phosphoglyceride conjugates of nucleoside analogs constitute another series of phospholipids. Although more attention has been directed toward nucleoside diphosphate diglycerides that incorporate a pyrophosphate linkage (see Section V), nucleoside monophosphate-linked L-diacylglycerols, precursors of certain cell membrane constituents, have shown biological activity. The corresponding phosphodiesters of various anticancer nucleosides (including araC, 5F-araC, 5F-Urd, 5F-dUrd, neplanocin) have been studied.[24,30,31] Some of them produced a significant increase in the life span of mice bearing P388 leukemia and were much more effective in these assays than the parent nucleosides. Phospholipid conjugates have also been developed with antiviral nucleosides like AZT.[32,33] *In vitro*, phosphatidyl-

AZT inhibited HIV replication in several cell lines following a series of catabolic reactions that could begin with phospholipase hydrolysis of the fatty acid esters and phosphodiesterasic degradation of glycero-3-phospho-5'-AZT to AZT and/or AZTMP followed by anabolic phosphorylation to AZTTP.[34,35] However, a phospholipid conjugate of acyclovir did not show significant activity against a TK⁻ HSV-1 strain,[36] and it was observed that *in vitro* phospholipase D converted the phosphatidyl derivative of 5-fluorouridine into its nucleoside parent.[37]

Several strategies involved nucleoside monophosphate-linked to other carriers that could induce internalization of their ligand *via* cell surface carbohydrate-specific receptors. These glycosylated carriers could be neutral polymers bearing mannosyl residues,[38,39] carbohydrates like D-glucose, ethyl D-mannopyranoside,[40,41] or sialic acid derivatives.[42]

The potential use of dinucleoside phosphodiesters has been investigated in anticancer chemotherapy. Such dimeric phosphodiesters of 5F-dUrd[43,44] and araC[45–47] have been studied, but none of them was more active than the nucleoside parents. In contrast, bis(6-mercaptopurine-9-β-D-ribofuranoside)-5',5'8'''-monophosphate (bis(MPR)P) exhibited significant activity against the 6-mercaptopurine-resistant human epidermoid cell subline (Hep N°2/MP),[16] whereas other antimetabolite dinucleoside phosphates were found to be no more effective than the parent drugs against sensitive and resistant neoplasms.[48] The concentrations of bis(MPR)P that were active against another MP-resistant sublines were considerably higher than these reported to inhibit Hep N°2/MP.[49,50] Synthesis and biological evaluation of dinucleoside phosphodiesters incorporating antiviral nucleosides have also been carried out,[13,20,51] but no evaluation against TK⁻ strains has been reported.

III. THE PHOSPHOTRIESTER APPROACH

Nucleoside phosphotriester prodrugs have also been extensively considered for NuMP delivery. As shown in Figure 3, one can conceive mononucleoside or dinucleoside phosphotriesters. Such neutral species are expected to easily cross the cell membranes, but their selective conversion to the corresponding NuMP will depend on various limiting factors. Phosphotriesters must first be transformed to the corresponding phosphodiesters before considering any transformation to the expected NuMP. But, since no phosphotriesterase activity has been reported in serum or inside the cells, this transformation must be a selective chemical process. This is the key problem that will partly condition the success of the phosphotriester prodrug approach.

As for the phosphodiester prodrug approach and for the same reasons, the phosphotriester has to be stable enough in cell culture medium before being taken up by the cells. We have thus to face a contradictory situation: the phosphotriester must be stable in culture medium but it must be decomposed selectively into the cytosol, the pH of both media being about 7.2.

Mononucleoside phosphodiester

5',5'—Dinucleoside phosphodiester

3',5'—Dinucleoside phosphodiester
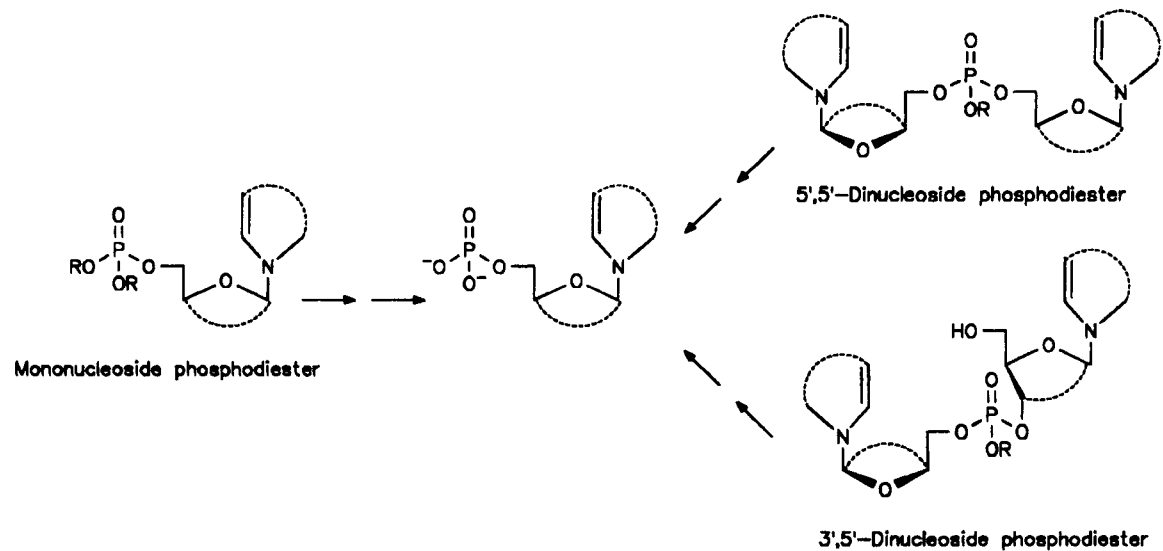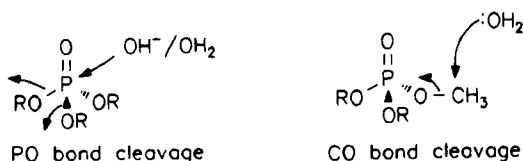
Figure 3.

Figure 4.

Considering the phosphotriester chemical hydrolysis process (Figure 4), it is well established that both P(V) and C alpha atoms are vulnerable to a nucleophilic attack.[52,53]

In basic medium, hydroxyde ions may attack the P(V) atom and hydrolysis will proceed through P-O bond scission. The same process may also arise at slightly basic or neutral pH, provided the phosphotriester is substituted by good leaving groups.

Since selectivity of elimination depends on the differences in the pK_a of the leaving groups, the mechanism of decomposition of nucleoside phosphotriesters that incorporate various aryl phosphate protecting groups has been studied in relation to the pK_a of the corresponding phenols.[22] As an example, the $t_{1/2}$ of the dinucleoside phosphotriester presented in Figure 5 was 6 hours in buffered aqueous solution at pH 7.7 (pK_a of paranitrophenol group 7.2), whereas the related mononucleoside phosphotriester of thymidine (Figure 5) was 50% hydrolyzed in 9.8 hours at the same pH. Both compounds were reported to give paranitrophenol as the sole elimination product. Note that the mononucleoside phosphotriester presented in Figure 5 exhibited in culture medium at 37°C a $t_{1/2}$ of 2.1 hours with further formation of the expected diester, of the corresponding 5'-mononucleotide and of the nucleoside.[54] The last two compounds were not formed in the absence of serum and presumably resulted from sequential phosphodiesterase and phosphatase ac-
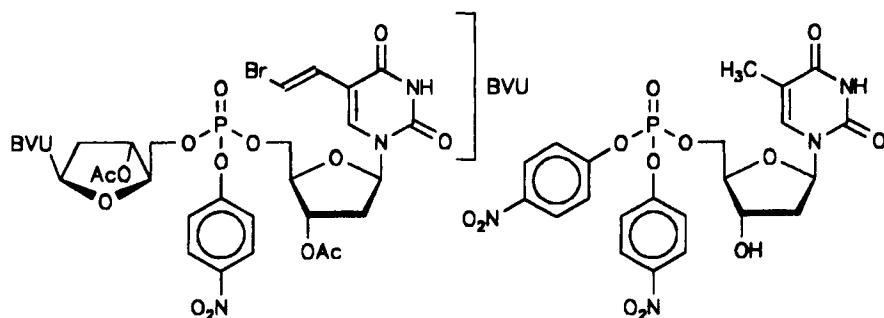
Figure 5.
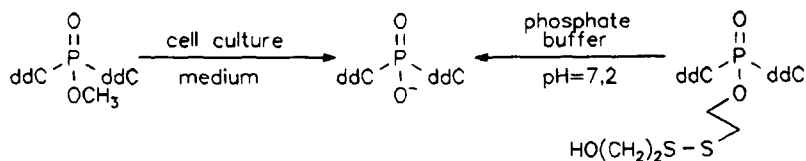
Figure 6.

tivities. This finding confirms again that degrading enzymes are still present in culture media. Appropriate leaving groups could be eventually envisaged in the prodrug approach, provided that the extracellular $t_{1/2}$ of the phosphotriester is sufficiently high to allow at least partial penetration into the cell. However, the eventual intracellular enzymatic hydrolysis of the formed phosphodiester must be further considered.

At neutral pH, a specific water nucleophilic attack on C alpha may arise with subsequent selective C-O bond fission. Soft nucleophiles also dealkylate phosphotriesters through C-O cleavage. Such reactions are typical SN_2 processes and show a clear preference for dealkylation of Me > Et > R_2CH.[52]

This implies that in culture medium alkylphosphotriesters may be slowly hydrolyzed by a pure chemical process. As a typical example, the 5,'5'-ddC methylphosphotriester (Figure 6) is slowly and selectively decomposed to the corresponding 5',5'-phosphodiester upon incubation in culture medium (6% of decomposition in 5 days).[12] For the same reasons, the 5',5'-ddC dithiodiethanolphosphotriester (Figure 6) presents a $t_{1/2}$ of 56 hours in phosphate buffer pH 7.2. One must point out that water nucleophilic attack on C alpha is specific to phosphotriesters and does not seem to occur on anionic phosphodiesters.[55]

The phosphotriester prodrug approach was considered by R. T. Walker's group in a series of publications dealing with some dinucleosidyl-5',5'-arylphosphotriesters.[22,56] The nucleoside moiety was of different types, from (*E*)-5-(2-bromo-vinyl)-2'-deoxyuridine (BVDU), acyclovir, to various dideoxynucleosides. As aryl protecting groups, the 4-(methylthio)phenyl and 4-(methylsulfonyl)phenyl were selected. It was indicated that the sulfonylaryl triester of BVDU (Figure 7) was
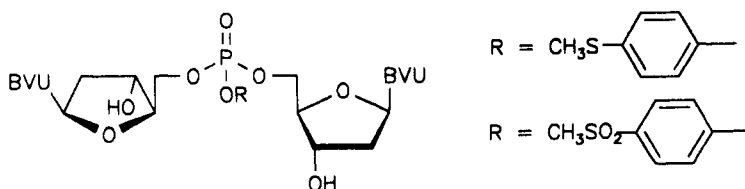
Figure 7.

stable in human serum and was chemically hydrolyzed to the corresponding dinucleoside phosphodiester with a $t_{1/2}$ of 17 hours at pH 7.7 (buffered aqueous solution). When subjected to enzymatic hydrolysis (data not shown), this phosphodiester was a substrate for snake venom phosphodiesterase and for the enzymes present in human serum to give BVDU as the final product.

It was further shown that the antiviral spectrum and potency of the BVDU and acyclovir dinucleoside phosphotriesters were remarkably similar to those of their parent nucleosides, suggesting that they act as a nucleoside prodrug, although the unexpected toxicity of one of the 4-(methylsulfonyl)phenyl triesters could also be explained by some intracellular BVDUMP liberation.[22] Of the dideoxynucleoside phosphotriesters, only those corresponding to 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T) and 2′,3′-dideoxyadenosine (ddA) had significant activity, their selectivity index being of the same order as the parent nucleosides. Nevertheless, an uptake process of the phosphotriesters was alleged.[56]

For the last 5 years in several publications, McGuigan et al. have proposed some transitory phosphate protecting groups of different types (such as alkyl, aryl aminoacids, etc.) with the aim of reaching structure–activity relationships.[57,58] Bioactive nucleosides such as AZT, ddC, d4T, 3′-fluoro-3′-deoxythymidine (FLT), araC, and 1-β-D-arabinofuranosyladenine (araA) were used and the corresponding antiproliferative activities were reported. All of the phosphotriesters were less potent than the parent nucleosides in various cell lines. No stability studies were reported, which makes those data very difficult to interpret in terms of decomposition pathways. Very recently, the same authors reported that nitroarylphosphotriester derivatives of AZT were inactive in TK⁻ cell lines. This implies that those compounds are not able to deliver the nucleotide intracellularly and that they act as a nucleoside delivery depots.[59] This result is not surprising since the inability of nucleoside arylphosphotriesters to act as sources of intracellular NuMP had been previously reported.[54]

In a series of publications, Huynh-Dinh et al. proposed to evaluate a series of nucleoside phosphotriesters structurally related to the dolichylphosphate structure, as a transport system to further deliver the NuMP.[40,41,60-62]
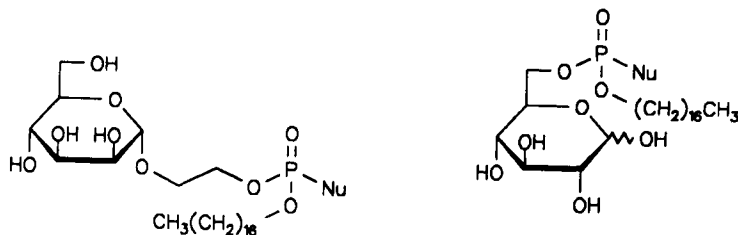
Figure 8.

In this respect, various nucleoside phosphotriesters bearing a lipidic chain and a glycosyl residue have been synthesized (Figure 8). Interaction of such phosphotriesters with large unilamellar vesicles was studied by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy. It was thus shown that these neutral molecules are transported through this membrane model in contrast with their corresponding phosphodiesters.[41,60,62]

Farquhar and Plunkett proposed to use acyloxymethyl bioreversible phosphate masking groups,[15,63,64] and it was expected that the corresponding mononucleoside phosphotriester could revert intracellularly to the parent NuMP. This implies a cleavage of the acyloxyl function by carboxylate esterase, followed by spontaneous formal elimination through a selective C-O bond breakage process (Figure 9).

After some preliminary studies on the enzymatic stabilities of various acylesters,[63] the *tert*-butyl group was selected and the corresponding pivaloyloxymethyl (POM) phosphotriesters of 5F-dUrd and ddU were synthesized.[15,63,64] Although the distribution of the activating carboxyesterases is ubiquitous (they can be found in the blood, liver, and tissues of most animals), it was unambiguously shown that under *in vitro* evaluation conditions the NuMP was intracellularly delivered.[64] As a proof, ddU was selected as a model (this compound is inactive because it is not metabolized to ddUMP), and the corresponding bis(POM)ddUMP was shown to present an anti-HIV activity in two human T cell lines. The metabolism studies fully corroborated the ddUMP delivery and its further anabolism to ddUTP.[64] In addition, decomposition studies of the corresponding bis(POM)AZT in culture medium and in total cell extracts were in total agreement with the expected mechanism.[65] The corresponding acetoxymethylester of adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) were later used with success to increase the intracellular delivery of such phosphate-containing second messengers.[66] It is noteworthy that formaldehyde, as a by-product in the elimination pathway of POM, was reported at the corresponding concentrations to be of little toxicity, at least in the short term.

A related approach, involving the use of acyloxybenzyl bioreversible phosphate protecting groups of AZTMP, was further reported by Glazier et al.[67] (Figure 10, Series A), and by Thomson et al.[68] (Figure 10, Series B).

For both series the same decomposition pathway was expected: esterase catalyzing the removal of the 4-acyl group, and the resulting electron-donating 4-hydroxy substituent then promoting cleavage of the benzyl-oxygen bond (selective C-O bond breakage process) to release the phospho anion, together with a short-lived intermediate with 4-hydroxybenzyl carbonium ion character.

The phosphotriesters corresponding to series B were evaluated for anti-HIV activity. With the exception of the pivaloyl substituted one, all of the phosphotriester compounds exhibited antiviral activities comparable to that of AZT. However, they were all ineffective in preventing HIV-1 infection of a kinase-deficient T cell line. It was therefore suggested that the compounds underwent metabolism to AZT in the culture medium.[68] However, toxicities of these compounds, which depended

Nu = 5–FdUrd , ddU , AZT

Figure 9.

Series A : R_1=C$_2$H$_5$COCH$_2$; R_2=CH$_3$

Series B : R_1=H ; R_2=CH$_3$, nC$_3$H$_7$, iPr , tBu
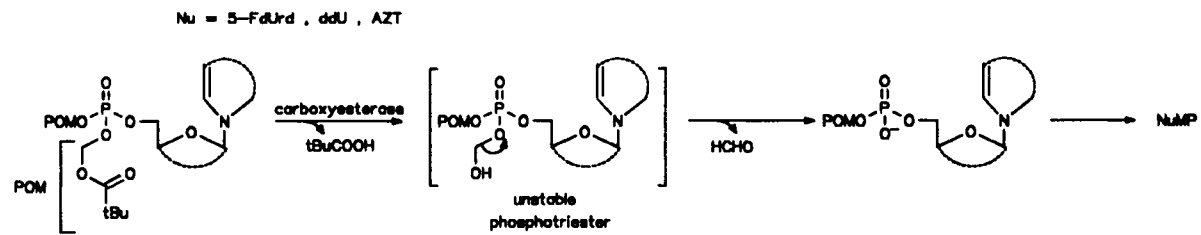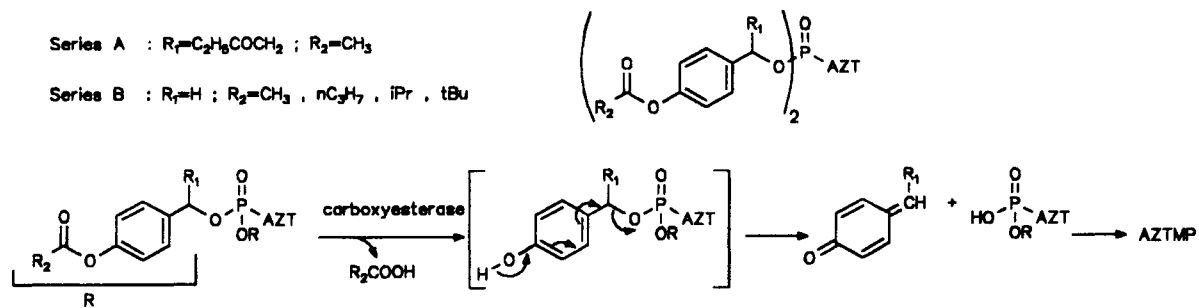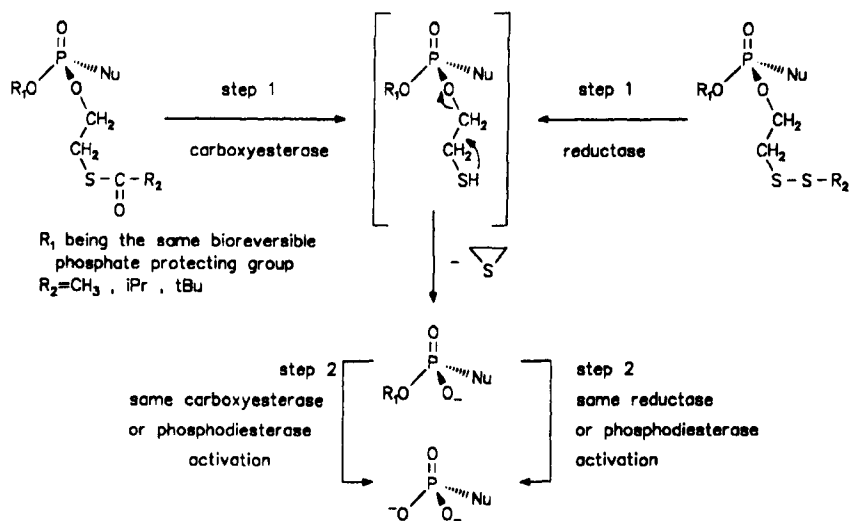
Figure 10.

Figure 11.

on the cell line, were in several instances substantially greater than that of the parent nucleoside.[67]

Recently our group reported on a related approach involving carboxyesterase or reductase activation, but with episulfide elimination.[12,14,55,69,70] In fact, the rationale behind the use of bioreversible phosphate masking groups is to obtain a transitory unstable phosphotriester that will spontaneously decompose to the corresponding phosphodiester.

In this respect, it was proposed to promote the enzymatic formation of a thioethanol transitory phosphotriester that will spontaneously eliminate episulfide (Eckstein phosphorothioate sequencing method) through a selective C-O bond breakage mechanism, as shown in Figure 11. Such phosphotriesters have been synthesized with several models, including ddU, AZT, and 9-(2-phosphonyl-methoxyethyl)adenine (PMEA, Figure 17). It was unambiguously demonstrated that this approach leads to intracellular NuMP delivery,[55,69] and kinetics of decom-position in various media fully corroborated the expected mechanism of the phosphotriester decomposition. It is noteworthy that the overall rate for NuMP delivery depends on step 1, which could be easily modulated according to the nature of the R_2 group, and step 2, which relies on competitive hydrolysis of the phos-phodiester to NuMP through either phosphodiesterase and/or the same mechanism as step 1, depending on the medium (Figure 11).[55]

Among the various explored bioreversible protecting groups, the S-acetyl-thioethanol (MeSATE) seemed to be well adapted to *in vitro* intracellular delivery of NuMP. When applied to ddA, the corresponding bis(MeSATE) phosphotriester

has been shown to be more active against HIV (by three orders of magnitude) than the parent nucleoside in various cell lines, which makes this compound more active than AZT.[70]

IV. THE HYDROGEN PHOSPHONATE APPROACH

Nucleoside-5′-*O*-hydrogen phosphonates (H-phosphonates), which are the tautomeric forms of nucleoside 5′-phosphites (Figure 12), have been considered as potential antiviral agents. It was postulated that they may penetrate the cell membrane (being weakly acid) and may then be oxidized to their corresponding 5′-monophosphates.

Several years ago it was reported that the triethyl ammonium salt of araA H-phosphonate was active against several DNA viruses but to a lesser extent than the parent nucleoside.[71] The same was then observed for an anti-HIV 4′-substituted nucleoside analog.[72]

On the other hand, in a series of publications dealing with H-phosphonate derivatives of numerous dideoxynucleosides, it was shown that a number of H-phosphonate derivatives exhibit high antiviral activities with selectivity close to (or higher than) that of the corresponding nucleosides.[73-75] All of the phosphonates initially showing activity were derivatives of active nucleosides, and because of their nature (salts of weak acids), the samples were found to contain small to significant amounts of their parent nucleosides.[73] After rigorous purification, all compounds, except for the H-phosphonates of AZT and FLT, showed diminished activity. It should be noted that the ddA derivative seemed to be slightly more active than the parent nucleoside.[73,76] It was proposed that H-phosphonates of modified nucleosides permeate cell membranes without preliminary hydrolysis and not as free nucleosides.[73,77] The possibility of a different metabolism or prodrug mechanism for these nucleoside H-phosphonates remains to be proved.[73] For our part, we have demonstrated that the H-phosphonate of AZT was rapidly metabolized to the parent nucleoside in cell culture medium (24 hr $< t_{1/2} <$ 33 hr) and even faster in CEM cell extract (41 min $< t_{1/2} <$ 69 min), the hydrolysis mechanism being unknown.[78] Intracellular transformation of the same compound was determined by another group in U937 cell lines (8 and 16 hours of incubation), and it was shown
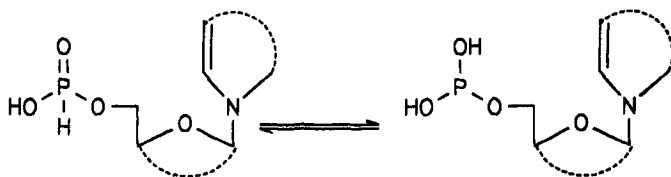
Figure 12.

that the H-phosphonate of AZT undergoes facile conversion to the corresponding AZTMP, the presence of AZT not being detected.[79] As pointed out, additional studies are required to elucidate the metabolic pathway(s) involved in this conversion,[79] but the fact that the AZT H-phosphonate showed no anti-HIV activity in TK⁻ cell line[78] suggests that thymidine kinase is necessary for nucleoside H-phosphonates to achieve their antiviral effect.

V. THE PHOSPHORAMIDATE APPROACH

Several 5'-phosphorodiamidate derivatives of 5F-dUrd have been evaluated as inhibitors of the growth of murine leukemia. The 5F-dUrd 5'-phosphorodiamidate was the most active compound.[80,81] To evaluate the ability of phosphorodiamidate nucleosides to generate the NuMP in mammalian cells, the corresponding *methyl*-³H-labeled thymidine derivative has been synthesized. The incorporation of radioactive deoxythymidine into DNA of cultured cells in which thymidine phosphorylation was blocked (genetically or by an enzyme inhibitor) has been studied.[54] The results provided no evidence that phosphorodiamidates act as a source of NuMP by cell permeation and subsequent metabolization. The synthesis and biological activity of some phosphorodiamidate of AZT have been also reported.[82,83] Carboxyl-protected amino acids or alkylamines were condensed with the 5'-phosphorodichloridate of AZT to give the corresponding diamidates. Generally, these compounds were less potent than the parent nucleoside, and no anti-HIV activity in TK⁻ cell lines was reported.

Cyclic phosphoroamidate derivatives of some nucleoside analogs (Figure 13, X = NH) have been extensively studied in relation to the metabolic oxidation and subsequent degradation of the anticancer drug cyclophosphamide.[84–86]

The putative mechanism for the *in vivo* hydrolysis of six-membered cyclic phosphoramidates involves biotransformation by hepatic P-450-dependent mixed-function oxidases to give the 4-hydroxy analogs (Figure 13, X = NH). It was anticipated that these compounds could penetrate into cells by passive diffusion and then undergo spontaneous ring opening to yield the acyclic tautomers, which would subsequently dissociate with elimination of acrolein to give the corresponding phosphoramidate.

These cyclic phosphoramidates were resistant to degradation by 5'-nucleotidase, alkaline phosphatase, venom phosphodiesterase, and crude snake venom. But none of them were significantly biotransformed when incubated with mouse hepatic microsomal preparations in the presence of an NADPH-generating system.

When administered intraperitoneally, the cyclic phosphoramidate of 5F-dUrd was nearly as effective as 5-fluorouracil at prolonging the life span of mice with leukemia. However, much larger doses of this derivative were required for optimal activity, and it was not active against a 5-fluorouracil-resistant tumor.[85]

The corresponding cyclic phosphotriesters of 5F-dUrd or araA (Figure 13, X = O) were, respectively, only marginally effective[85] and inactive[86] against P-388
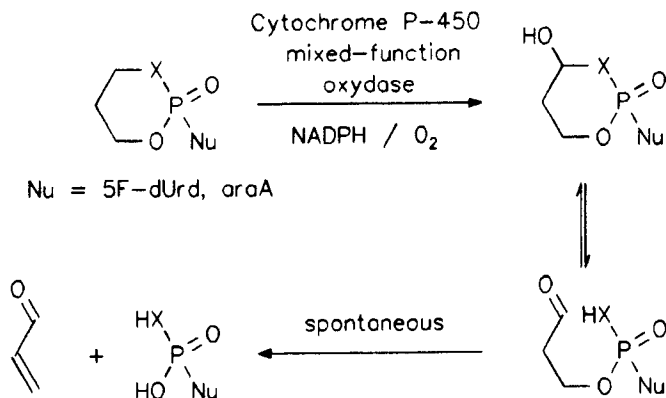
Figure 13.

leukemia. Various substituents in the 5 position of the 1,3,2-dioxaphosphacy-clopentane ring of the phosphotriester of 5F-dUrd have been introduced,[87] and one of them (the *gem*-difluoro derivative) was nearly as potent as the nucleoside parent in its inhibitory effect on murine leukemia L1210 cells.

The biological properties of five-membered cyclic phosphoramidate (Figure 14, X = NCH$_3$) derivatives of nucleoside analogs have also been studied.[88–90] Like the corresponding phosphotriesters (X = O), which are easily chemically hydrolized (i.e., in a few minutes at physiological pH at room temperature),[91,92] these cyclic phosphoramidates are hydrolyzed under physiological conditions without the necessity of enzyme activity. *In vitro*, these derivatives showed potent antitumoral or antiviral activities, but they were not active against cells that were resistant to the respective nucleoside analogs.[88,90]

Recently the synthesis and anti-HIV evaluation of some aryl phosphoramidate derivatives of AZT have been reported.[58,93] Several of these compounds (Figure 15) retain significant activity in TK$^-$ cell lines, supporting the hypothesis that such AZT phosphate derivatives could exert their biological effects *via* some intracellular AZTMP release.

Nu = 5F–dUrd, acyclovir, BVDU
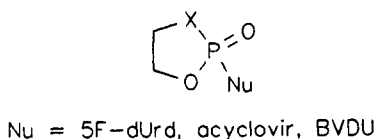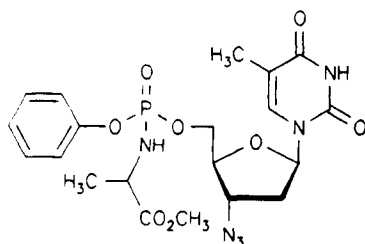
Figure 14.

Figure 15.

VI. THE PYROPHOSPHATE APPROACH

A variety of analogs of cytidine diphosphate diglyceride (CDP-DG), a naturally occurring intermediate in anionic phospholipid biosynthesis, have been synthesized and evaluated *in vitro*. A potential advantage of this type of conjugate is the release of NuMP, as a result of substitution of the liponucleotide in the metabolism of CDP-DG.[94]

Ara-C conjugates of biologically active thioether (1-*S*-alkyl) phospholipids have demonstrated superior antitumor activity against both animal leukemia and solid tumor models.[95] Among them, Cytoros (Figure 16) has shown significant therapeutic effects on human colorectal[96] and PSN-1 paricreatic cancer xenografts in nude mice.[97]

Diphosphate diglycerides of antiviral nucleoside analogs (AZT, ddC, ddT, and acyclovir) showed activity *in vitro*.[98–102] A pyrophosphate linkage is necessary for the phospholipid prodrug to generate antiviral activity in cells infected with mutant viruses deficient for TK. Thus, contrary to nucleoside monophosphate-linked L-diacylglycerols (see Section II), the corresponding diphosphate diglyceride derivatives of ddT and acyclovir showed activity in TK⁻ cell lines, and it was demonstrated that a mitochondrial pyrophosphatase cleaved these phospholipid prodrugs to the corresponding NuMP.[101]

Cytoros
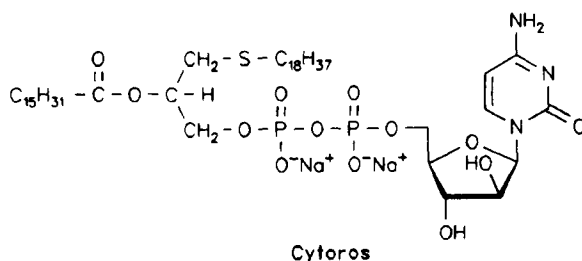
Figure 16.

VII.   THE PRODRUG APPROACH APPLIED TO NUCLEOSIDE PHOSPHONATES

Mononucleotide analogs bearing a P-C linkage have been intensively studied, and some acyclic derivatives such as PMEA and (*S*)-1-(3-hydroxy-2-phosphonyl-methoxypropyl)cytosine (HPMPC, Figure 17) display significant antiviral activity against several RNA and DNA viruses.[103,104]

Such compounds are isopolar with mononucleotides, resist enzymatic dephosphorylation, and are converted in cells by cellular nucleotide kinases or by 5-phosphoribosyl-1-pyrophosphate synthetase to their mono- and diphosphate derivatives, these latter inhibiting selectively viral polymerases.

Since the phosphonyl group exhibits a negative charge at physiological pH, the oral bioavailability and cellular uptake of these molecules are relatively poor.[105] In the hope of increasing the PMEA potency, its phosphonate functionality has been masked with pivaloyloxymethyl (POM, Section III) groups (Figure 18).[106] Compared with the parent compound, the bis(POM) ester of PMEA showed *in vitro* enhanced antiviral activities.[106,107]

The uptake enhancement was corroborated later by cellular metabolism studies with radiolabeled compounds. However, it was also shown that the cytotoxicity of bis(POM)PMEA, which is relatively unstable at physiological pH ($t_{1/2}$ = 4 hr),

PMEA   HPMPC

Scheme 17.

bis(POM)PMEA   bis(DTE)PMEA
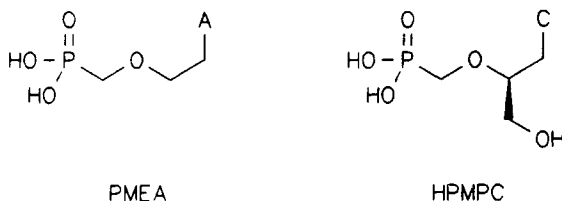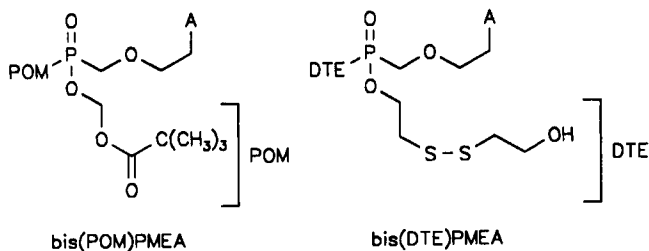
Scheme 18.

breaking down to the mono(POM) derivative,[107] was enhanced to various degrees as compared to the patent compound.[107–109]

PMEA was also transformed to the corresponding bis(DTE) ester (Figure 18). When evaluated for its anti-HIV activity in various cell lines, this compound exhibited *in vitro* a more potent antiviral effect by one or two orders of magnitude than the parent PMEA.[55] It was postulated, like for the bis(POM)PMEA, that such an increase of activity may be due to an enhanced uptake.

VIII. CONCLUSION

The major prerequisite for most antiviral nucleosides is their first enzymatic phosphorylation step. Thus, the design of a suitable nucleotide delivery form, presenting appropriate toxicology and pharmacology parameters, is a key problem. Solving the pronucleotide approach is of fundamental importance for the design of any bioactive nucleotides whose biological effects are limited by poor uptake, absence (or mutation) of the appropriate activating enzymes, and catabolism. Appreciation of these factors, which are species, tissue, and cell specific, as well as cell cycle dependent, reinforces the interest in solving the nucleotide delivery problem.

Based on previous *in vitro* published data, it appears that one must be very careful in distinguishing a pronucleotide from a pronucleoside, particularly when a bioactive nucleoside is used.

If one succeeds in *in vitro* intracellular delivery of an NuMP, numerous consequences may arise in term of metabolism and of efficiency as compared to the parent nucleoside. Some preliminary *in vitro* data on specific nucleosides such as ddU,[55,64,69] acyclovir,[98] and ddA[70] as well as PMEA[107] confirm this point.

However, the toxicological consequences of the pronucleotide approach must be carefully evaluated as the promoiety liberation, and the variation in NuMP intracellular concentration may provide some undesirable side effects. Furthermore, because of a different distribution of some degradative enzymes (i.e.. phosphodiesterase, phosphatase, etc.), a well-established *in vitro* pronucleotide may not be fully adapted to *in vivo* intracellular delivery. However that may be, as compared to the parent nucleoside, one can expect a modification of the drug bioavailability, which may have some beneficial consequences. As an example, the bis(POM)PMEA can be considered *in vitro* as an intracellular delivery form, but *in vivo* it may more likely act as a depot form that increases PMEA bioavailability after oral administration without necessarily ensuring intracellular penetration of the monoester.[107]

In conclusion, a therapeutic approach based on the use of nucleotide prodrugs could represent in the future an additional curative response to cancer or viral infections. One must keep in mind when pronucleotides are designed that these compounds must be appropriate for *in vivo* experiments.

ACKNOWLEDGMENTS

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DISCOVERY AND DESIGN OF HIV PROTEASE INHIBITORS AS DRUGS FOR TREATMENT OF AIDS

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ABSTRACT

The protease encoded within the *pol* gene of human immunodeficiency virus (HIV) is essential for maturation of the newly budded virion to an infectious viral particle. For this reason, inhibitors of the HIV protease have been developed as possible drugs for treatment of acquired immunodeficiency syndrome (AIDS), a condition associated with HIV infection. The viral protease is a paradigm for rational drug design. It is a member of a well-characterized mechanistic set of proteases, the aspartyl enzymes, and therefore much is known by inference regarding its structure and mechanism. Yet, it is also unique among all known proteases in having a dimeric structure, and accordingly, it offers a selective target for drugs in humans. The monomeric units, identical, 99-residue polypeptides, each contribute symmetrically to the catalytic site, and the enzyme is a C_2-symmetric dimer. At the time of this writing, hundreds of structures have been solved crystallographically for the HIV-1 protease, both alone and in complexes with a wide spectrum of inhibitors, so the enzyme ranks among the best understood in terms of both structure and mechanism. The protease is also unique in its specificity, being able to hydrolyze almost any peptide bond, given an optimal distribution of amino acids in P_4 through P_4', and accessibility to the bond in question. This specificity has been the basis for early development of protease inhibitors with noncleavable transition state inserts replacing the scissile bond pair. However, if an HIV protease inhibitor is to be successful as a drug for treatment of AIDS, it must not only be potent in blocking enzyme activity, but it must satisfy the usual criteria that define a useful therapeutic agent. The last 5 years have witnessed a transition from peptide-like inhibitors that are generally marginal as drugs because of poor solubility and/or bioavailability, to smaller,

nonpeptide, organic molecules with enhanced pharmacokinetic properties. The process of protease inhibitor development has encompassed everything from serendipitous, classical screening approaches to de novo design. The latter achievement has been possible through an iterative cycle of crystallographic analysis, computer-assisted refinement and prediction, organic synthesis, and biological assays. In fact, this whole process of discovery of drugs designed to target the HIV protease represents a powerful alliance among numerous disciplines, including virology, biochemistry, molecular biology, X-ray crystallography, computer modeling, medicinal chemistry, pharmacology, and applied medicine. This chapter focuses on this process, highlighting those protease inhibitors that have been brought forward for clinical trials, and providing some early results in the clinics. Some discussion is also included of mutations in the protease, which have been documented in viral strains that show resistance to protease inhibitor drugs.

I. INTRODUCTION

Despite the deployment of a worldwide effort over the past decade to come to grips with the deadly scourge of acquired immunodeficiency syndrome (AIDS), there is no cure for the disease, and it can be argued that, at present, there is no effective therapy to prolong the life of infected persons. It is generally held that a retrovirus, human immunodeficiency virus (HIV), is the causative agent in AIDS, and the virus continues to be a prominent target in the search for AIDS therapeutics. Progress toward discovery of an effective vaccine against HIV, or a cure of AIDS, has been handicapped by the complexity of the disease. Although a wealth of information is available with regard to the molecular biology of the virus, many aspects of infection and progression to disease remain obscure, and the crippling effect of the virus on the host immune system is yet to be understood in a way that might lead to definitive therapeutic intervention.

Because so much is known about the life cycle of the virus and its mechanisms of replication and maturation, antiviral drugs have been a major focus of attention.[1] Retroviruses, including HIV, possess a number of unique enzyme targets that are not present in the host. One that has been studied intensely is the reverse transcriptase (RT), an enzyme essential for making a DNA copy of the viral RNA message, which can then be inserted into the host genome. AZT (Zidovudine, 3'-azido-3'-deoxythymidine) and related nucleoside derivatives, suicide substrate precursors of RT, are still the only approved drugs for AIDS victims, despite the toxic side effects of these compounds[2] and the fact that, within a few month's time, forms of the virus resistant to these drugs become prominent.[3-5] Development of nontoxic, non-nucleoside RT inhibitors has led to safe and highly potent drugs with excellent pharmacokinetic properties.[6] These have shown some efficacy in the clinic, but only for a short time; again, the rapid selection and replication of resistant variants leads to escape from the drug effect.[5-8] Diabolically, the infidelity of RT itself in copying its genetic message contributes in a major way to the generation of diverse

		HIV-1	HIV-2	SIV
I	p17-p24	...SQNY ↓ PIVQ...	...GGNY ↓ PVQH...	...GGNY ↓ PVQQ...
II	p24-p1	...ARVL ↓ AEAM...	...ARLM ↓ AEAL...	...ARLM ↓ AEAL...
III	(p24-p1)-p9	...ATIM ↓ MQRG...	...PFAA ↓ AQQR...	...PFAA ↓ AQQK...
IV	p9-p6	...PGNF ↓ LQSR...	...PRNF ↓ PVAQ...	...PRNF ↓ PMAQ...
V	TF -PR	...SFNF ↓ PQIT...	...GLAA ↓ PQFS...	...GFAA ↓ PQFS...
VI	PR-RT	...TLNF ↓ PISP...	...SLNL ↓ PVAK...	...SLNL ↓ PIAK...
VII	RT-RN	...AETF ↓ YVDG...	...AQTF ↓ YTDG...	...EETY ↓ YVDG...
VIII	(RT-RN)-IN	...RKIL ↓ FLDG...	...RQVL ↓ FLEK...	...RQVL ↓ FLEK...
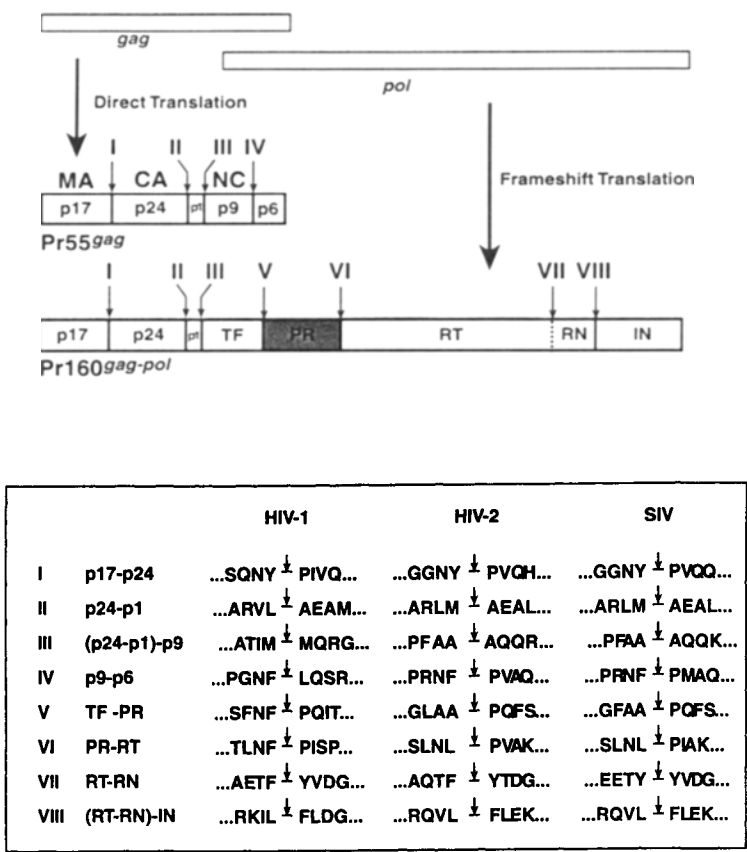
Scheme 1. *gag* and *pol* reading frames of HIV-1 and their polyprotein translation products Pr55^{gag} and Pr160$^{gag-pol}$ (**top**). Sites of cleavage by the HIV-1 protease and, for comparison, by the HIV-2 and SIV (simian immunodeficiency) proteases are indicated by small arrows (**bottom**). The amino acid sequences are in the one-letter code. *gag*, group specific antigen; *pol*, polymerase; MA or p17, matrix protein; CA or p24, capsid protein; NC or p9, nucleocapsid protein; TF, transmembrane protein; RT, reverse transcriptase; RN, ribonuclease; IN, integrase.

quasi-species. This problem notwithstanding, RT continues to be a prime target for antiviral development, and blood levels achievable for some of the nontoxic non-nucleoside RT inhibitors are orders of magnitude higher than needed to halt viral replication in cell culture. It may be that this high therapeutic index and/or combination therapy will help to improve efficacy of these drugs against mutant strains.

Another HIV enzyme that has proved to be a worthy target is the virally encoded protease that is responsible for processing of the *gag* and *gag/pol* polyproteins, an

essential step leading to viral maturation. The retroviral genome is divided into three large reading frames, namely, *gag*, *pol*, and *env* (not considered here). The *gag* open reading frame (1536 nucleotides) is translated directly into a 55-kDa precursor (Pr55^{gag}), which is a polyprotein comprising the nucleocapsid and core proteins of the virion (Scheme 1). The *pol* open reading frame (3045 nucleotides) is translated only as a *gag/pol* fusion polyprotein, Pr160$^{gag/pol}$, by virtue of a frameshift in the p6 region (Scheme 1). Both polyproteins are N-terminally myristoylated, a post-translational modification that is indispensable for their attachment to the host cell membrane. The HIV protease is absolutely essential for processing of the Pr55^{gag} and Pr160$^{gag/pol}$ polyproteins during the final maturation stage in the viral life cycle. Budded, immature viral particles that contain a catalytically inactive protease,[9] or one in which the protease is inhibited,[10] cannot undergo maturation to an infectious form. Based upon its DNA-derived protein sequence, the HIV protease was predicted from the start to be a member of the aspartyl proteases,[11] enzymes in which two aspartyl residues participate in catalysis. Prominent members of the aspartyl proteases include pepsin, renin, and cathepsin D, double-domain enzymes of about 35 kDa in which the N- and C-terminal halves are homologous. Each half contains one catalytic Asp residue in a highly conserved Asp-Thr/Ser-Gly sequence.[12] In the case of the HIV protease, there is only one Asp-Thr-Gly sequence in its relatively small 99-amino acid polypeptide chain; the catalytic entity, it was reasoned, had to be a dimer.[13] Subsequent X-ray crystallographic analysis of the HIV protease[14–16] bore out all of the inferences based upon knowledge of its sequence and its structural relationship to the aspartyl enzymes. At the time of this writing, there have been dozens of publications describing the structure of the HIV protease in the presence or absence of bound inhibitors, and the structures of many more enzyme:inhibitor complexes have been solved in the laboratories of pharmaceutical companies. This C_2 symmetrical aspartyl protease dimer appears to be unique to the retroviruses, and this lends credence to the idea that one could find or design drugs for the selective inhibition of the HIV protease in an infected person.

For several reasons, therefore, the HIV protease is an ideal target for a host of biochemical investigations, and a paradigm for drug design. The enzyme can be cloned, expressed, and purified in large quantities[16–22]; it can be manipulated genetically to probe structure–activity relationships[23–26]; and tandem "covalent dimers" have been produced by recombinant techniques.[27,28] Synthetic chemistry has allowed complete synthesis of the HIV-1 protease from both L-[29–31] and D-[32] amino acids. Finally, the enzyme is amenable to crystallization, and high-resolution structural information is readily available for probing inhibitor binding sites (e.g., see Wlodawer and Erickson[33] and references therein). This sophisticated level of structural information can be evaluated by current computer-assisted analytical programs to predict a course for rational chemical synthesis and testing of enzyme inhibitors in an iterative process of drug refinement.

This chapter will focus primarily upon the HIV type 1 (HIV-1) protease as a target for inhibitor design, with special emphasis on compounds with potent antiviral

activity that have been brought forward into clinical trials for treatment of AIDS. An account of some early peptide-like protease inhibitors that resulted, by and large, from knowledge of the specificity of renin and past experience with renin inhibitors will be followed by a discussion of inhibitors, both peptide and nonpeptide, that resulted from screens or from de novo design principles. Many review articles have appeared on the subject of the HIV-1 protease and its inhibitors, and the reader may wish to refer to these[33–38] for recent accounts and further references.

II. CATALYTIC MECHANISM AND SPECIFICITY OF THE HIV PROTEASE

A. Catalytic Mechanism

As mentioned above, as soon as it was recognized that the HIV protease is a member of the aspartyl proteases, all of the mechanistic information gleaned from studies of the latter enzymes over the past several decades fell into place, by inference, for the retroviral enzyme. The hallmark of the aspartyl proteases is the presence of two catalytic β-carboxyl groups from the side chains of two aspartyl residues brought into close proximity by the protein fold. In the case of the dimeric HIV-1 protease, each monomer chain donates a single Asp residue at position 25 in the polypeptide chain. Residues in one of the chains are numbered 1, 2, 3, . . . 99, and residues in the other chain are numbered 1′, 2′, 3′, . . . 99′; thus one chain would have Asp_{25} and the other $Asp_{25'}$. This convention will be followed in this chapter, unless noted to the contrary.

Plots of V_{max} versus K_M give a bell-shaped curve from which pK_a values of 3.4 to 3.7 (acidic Asp) and 5.5 to 6.5 (basic Asp) have been calculated.[39,40] This would suggest that the HIV protease, like other members of this mechanistic set, participates in general acid-general base catalysis where one of the catalytic Asp residues is protonated and the other is not. The nucleophile in catalysis is, most likely, an activated water molecule (Figure 1). Based upon this information and on extensive ^{18}O-exchange experiments, Hyland et al.[39,40] proposed that the substrate is positioned between the two catalytic carboxyl groups so as to form a hydrogen bond between the carbonyl oxygen of the substrate and the proton on Asp_{25} (Figure 1). Here, the lytic water is in close proximity to the β-carboxylate of $Asp_{25'}$, which abstracts a proton from it (EAH′ in Figure 1). The hydroxide ion formed in the latter event attacks the carbonyl carbon of the scissile peptide bond and, simultaneously, Asp_{25} carries out protonation of the carbonyl oxygen. This gives the enzyme-bound amide hydrate (EXH complex) via the transition state (indicated as [ ] in Figure 1). Based upon ^{18}O-exchange, it appears that the formation and reversal of the EXH complex must be faster than its breakdown to products; i.e., the rate-limiting catalytic step is k_7. In this model,[39,40] formation of an acyl enzyme during catalysis is excluded. A similar mechanism was proposed earlier by Jaskolski et al.,[41] based upon structural analysis of an HIV-1 protease:inhibitor complex. In this case,
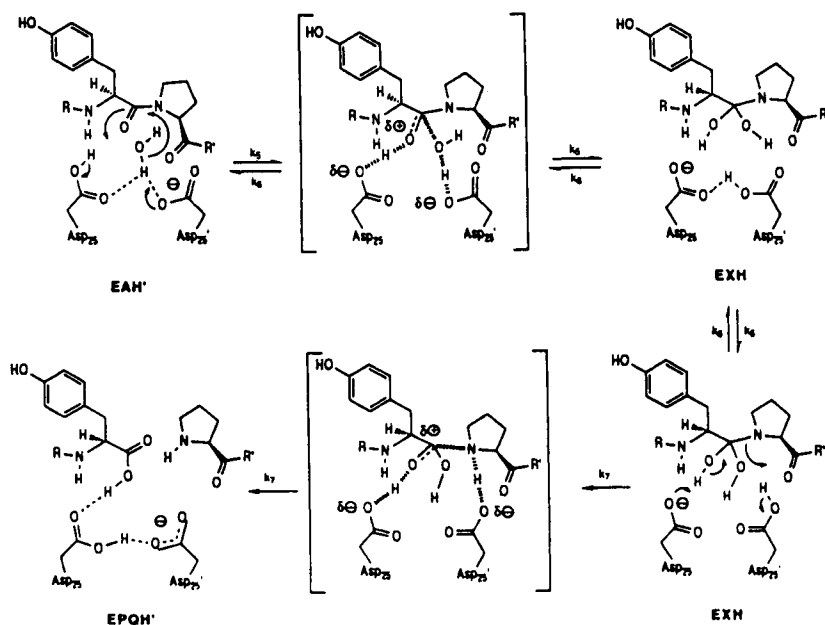
Figure 1. Chemical mechanism of peptide bond hydrolysis catalyzed by the HIV-1 protease as proposed by Hyland et al.[40] Reproduced with permission.

however, proteolysis is represented by a "one-step" process whereby the nucleophilic water molecule and the electrophilic acidic proton attack the scissile peptide bond in a concerted fashion.

These mechanistic inferences are highly relevant to drug design in that they support the idea of making HIV-1 protease inhibitors with so-called transition-state mimics as replacements for the scissile bond. Examples in the following narrative will give credence to this line of reasoning, which has led to powerful inhibitors of the protease with potent antiviral activity in which the scissile P_1-P_1' peptide bond of the substrate has been replaced by a nonhydrolyzable isostere with tetrahedral geometry.

B. Specificity

This discussion of the mechanism of HIV protease catalysis has dealt only with events involving the scissile peptide bond in the substrate as they relate to the two catalytic Asp residues and a nucleophilic H_2O molecule in the enzyme. However, this is only the last stage of a larger chemical process that involves recognition and binding of selected substrates containing sequences at least seven amino acids in length.[42] It is appropriate, as well, to highlight some of the features that are

prominent in substrates of the HIV-1 protease, because ideas about the design of enzyme inhibitors generally come from a sophisticated understanding of the substrate specificity of the enzyme target.

When the eight processing sites in the HIV *gag* and *gag/pol* polyproteins were deciphered, it immediately became apparent that the retroviral protease is versatile in its mode of substrate recognition (Scheme 1). Unlike most proteolytic enzymes that show a strong preference for a particular amino acid in P_1 or P_1' (notation according to Schecter and Berger),[43] the HIV-1 protease can readily hydrolyze a diverse set of peptide bonds, including those with Pro in P_1'. Exploitation of this latter aspect of protease specificity has led to highly selective and potent antiviral inhibitors of the HIV-1 protease; more details will be given in the following section.

We recently reported a compendium of 64 different octapeptides representing positions P_4 through P_4' in substrates that undergo cleavage by this enzyme,[44] and we have earlier defined the specificity of the HIV-1 protease as being cumulative.[45] By this we mean that substrates are recognized and bound by virtue of a cumulative set of contributions provided by interactions between the enzyme and each amino acid in positions P_4 through P_4' of the substrate. Accordingly, the HIV protease can cleave almost any peptide bond, as long as the enzyme has access to the site in question and the amino acids occupying P_4 through P_4' in the substrate are reasonably well accommodated in the protein subsites. In this view, cleavage at any particular site is dictated by many surrounding amino acids rather than by a single residue at P_1 or P_1'. Several algorithms for predicting sites of cleavage by the HIV-1 or HIV-2 proteases in proteins and/or peptides have been published.[45–47]

However, this idea of a generalized, cumulative specificity is subject to at least one major qualification. Although there appear to be few preferences for amino acids at any of the eight positions from P_4 to P_4', certain residues are excluded, namely Lys at any position from P_2 to P_2', and β-branched amino acids at P_1. Rosé et al.[48] were the first to show that insertion of a Lys in the P_2' position (in place of Gln_7 of the HIV-1 protease) blocked one of the major sites of autolysis in the enzyme at Leu_5-Trp_6. We have extended this idea to include insertions of β-branched amino acids at the P_1 positions of the other autolysis sites to see if these replacements might be sufficient, in and of themselves, to block cleavage at P_1–P_1'.[26] In so doing, we were in fact able to create a fully active mutant enzyme with unaltered specificity that was resistant to autolysis. Therefore, these single-point mutations were able to override the cumulative specificity of the protease. Other groups have tested the same concepts by analysis of peptide substrates modeled after processing sites in the viral polyproteins in which a variety of amino acids were substituted at positions from P_4 through P_4'.[49–51] The important point to note with regard to specificity is that the design or selection of inhibitors of the HIV-1 protease is open to a wide variety of structural possibilities.

III. THE THREE-DIMENSIONAL STRUCTURE OF THE HIV-1 PROTEASE

Because of the intense interest in the HIV-1 protease as a therapeutic target in AIDS, this enzyme has become one of the best understood in terms of both structure and function. As mentioned above, the HIV-1 protease is a homodimer composed of two interfacing monomer chains, each having 99 amino acid residues (Figure 2). The architecture is reminiscent of that seen in the well-characterized double-domain pepsin-like enzymes. However, the HIV-1 protease homodimer is perfectly C_2-symmetric in the absence of any ligands other than water; pepsin, renin, cathepsin D, etc., are not. As will be discussed later, the symmetry of the retroviral enzyme is lost upon binding of ligands.

The ability of the dimer to dissociate to inactive monomeric units is an important aspect of its function. Activation of the protease within the polyprotein format is designed to occur only after budding of the nascent immature viral particle, a process associated with concentration by several orders of magnitude. Premature activation within the infected cell would abort production of infectious virus. Darke et al.[53] have reported that the K_d of the HIV-1 protease is strongly pH dependent. Values of 0.75 nM and 3.4 nM at 30°C and 37°C, respectively, were determined at pH 5.5, where the enzyme is most active catalytically. Strong intermolecular interactions provided by a network of hydrogen bonds in the β-sheet made up of the N- and C-terminal segments of the two monomers account for much of the dimer stability. Remarkably, the four-residue C-terminal segment contributes 45% of the dimer interface and a considerable amount of stabilization energy.[54] It has been demonstrated experimentally[55] that a tetrapeptide corresponding to the four C-terminal amino acid residues in the polypeptide chain, Ac-Thr-Leu-Asn-Phe-OH, is able to bind to inactive protomers and prevent their association into the active protease dimer (dissociative inhibition) with a reported K_i of about 45 μM.

A. The Active Site

The active site cavity is formed by residues 8, 23, 25, 27–30, 32, 47–50, 53, 80–82, and 84.[56] These residues are highlighted in Figure 3, A (hydrophobic residues) and B (nonhydrophobic residues), and their relevance to inhibitor/substrate binding is discussed in the following narrative. The catalytic triads, Asp_{25}-Thr_{26}-Gly_{27} and $Asp_{25'}$-$Thr_{26'}$-$Gly_{27'}$, lie on the "floor" of the cavity with the catalytic Asp_{25} and $Asp_{25'}$ in near-coplanar orientation (Figure 2). Each triad is part of a loop that is made relatively rigid by a network of hydrogen bonds. Moreover, this loop in one monomer and that in the dyad-related mate are interlinked by four hydrogen bonds in a so-called fireman's grip configuration,[57] characteristic of what is seen in all aspartyl proteases.[58,59] The "ceiling" of the catalytic site is composed of a pair of six-amino acid segments (...Ile_{47}-Gly-Gly-Ile-Gly-Gly_{52}...) and the corresponding region, 47'-52', in the other monomer, which form the "flaps." The
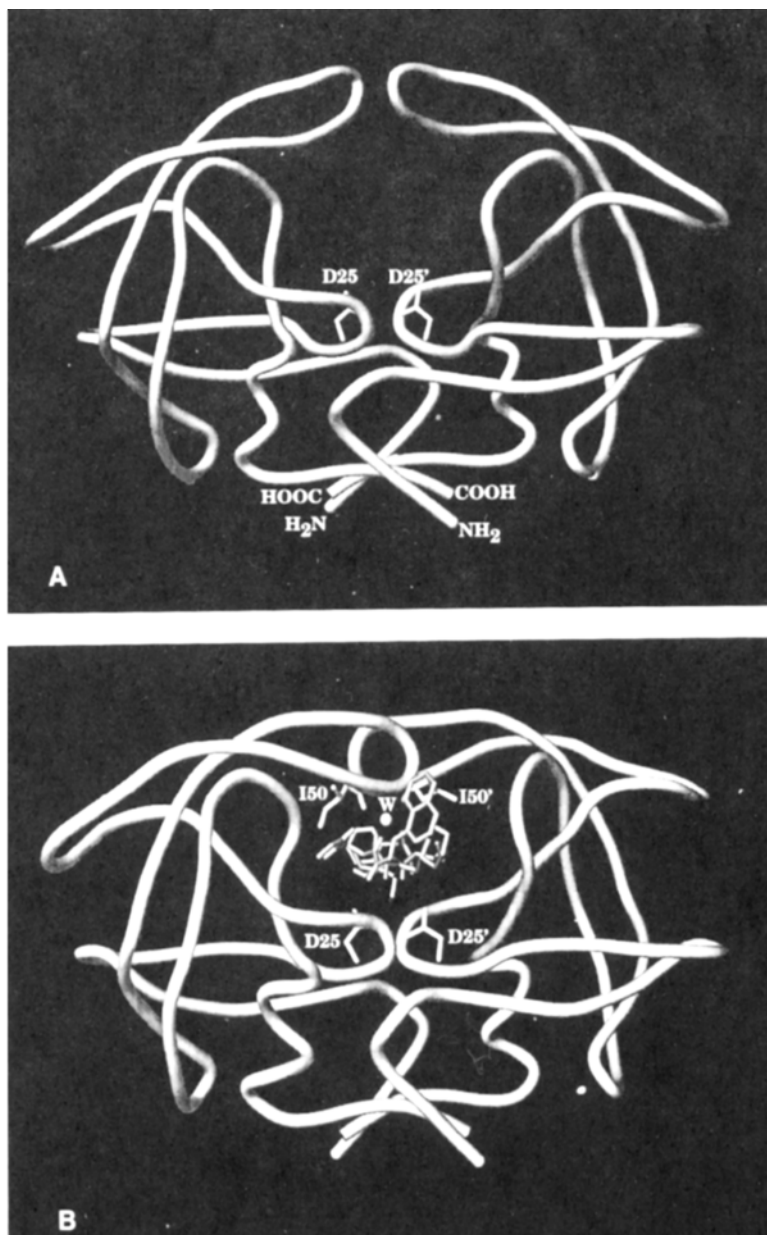
Figure 2. The 3D structure of dimeric HIV-1 protease. (**A**) Apoenzyme. (**B**) Enzyme complexed to an inhibitor (U-75875, Thanki et al.[52]); $D_{25}/D_{25'}$, catalytic aspartates; $I_{50}/I_{50'}$, isoleucine residues that coordinate the structural water molecule (W) via hydrogen bonds.
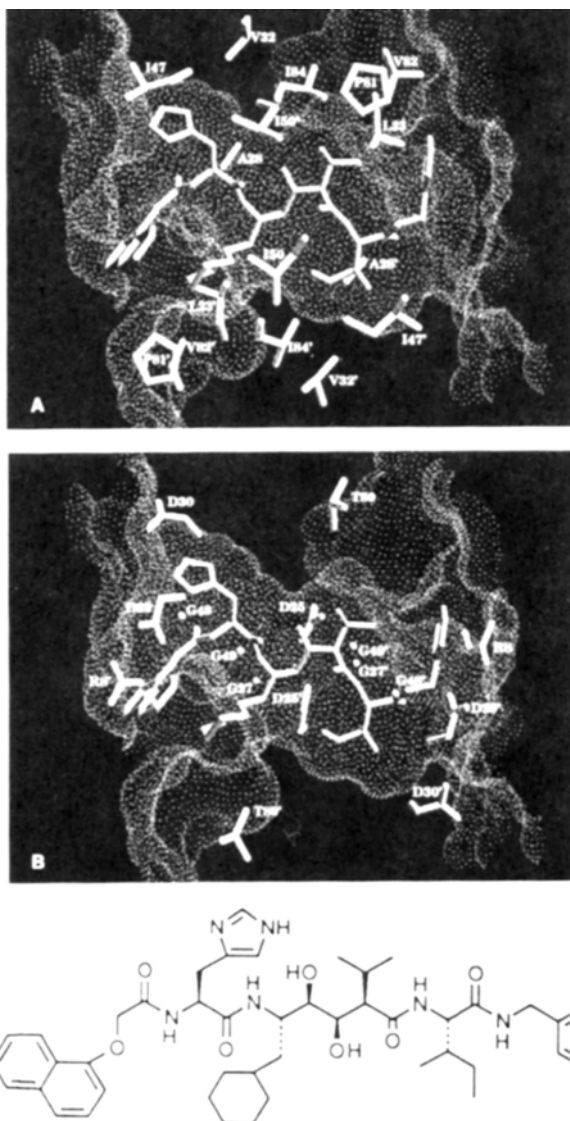
Figure 3. Amino acid residues at the active site of HIV-1 protease; the enzyme is complexed to the inhibitor U-75875 (grey color; chemical structure in **C**). Patterns are shown of van der Waals surfaces (white dots) and side chains (white tubes) of hydrophobic (**A**) and nonhydrophobic (**B**) amino acids that interact with the potent inhibitor U-75875. Since the major contribution of nonhydrophobic amino acids to inhibitor/substrate binding is via hydrogen bonds, details of the hydrogen bond network linking the inhibitor/substrate to the enzyme active residues are given in Figure 4. (**C**) The chemical structure of U-75875.

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flaps are flexible structures stabilized by a pair of hydrogen bonds between main-chain atoms of the residue located at the tip of the flap and its twin partner in the opposite monomer. The flaps cover the active site and play an important role in substrate and inhibitor binding. Pepsin-like enzymes have only a single flap with a different orientation.[60] Upon binding the inhibitor, the HIV-1 protease undergoes a conformational change that rotates the two monomers about 2° around a hinge axis located in the dimer interface; here the rotation axis is perpendicular to the view shown in Figure 2. The most striking structural feature of this change is the large motion of the flap regions. The flaps in the apoenzyme are in an open conformation (Figure 2A), but in the presence of bound ligand they shift toward the ligand as much as 7 Å (ends of flaps) to embrace it (Figure 2B). A water molecule with approximate tetrahedral coordination bridges the P_2 and P_1' carbonyl groups of the inhibitor to the amide groups of Ile_{50} and $Ile_{50'}$ of the flaps via hydrogen bonds. This water molecule is a unique feature of the retroviral proteases and is not seen in conventional pepsin-like enzymes. Another segment of the enzyme that undergoes substantial motion upon ligand binding is that encompassed by residues 77–82, which shifts more than 2 Å toward the inhibitor to further decrease the size of the active site pocket. The hydrophobic interactions between the two pairs of active site residues, $Pro_{81/81'}$, $Val_{82/82'}$, and inhibitor side chains at the P_1 and P_1' positions, are responsible for the shift. Grinde et al.[61] have shown the specific involvement of $Val_{82/82'}$ in substrate recognition. Moreover, as will be detailed later, mutations in the protease involving Val_{82} have been implicated in viral resistance to protease inhibitor-type drugs.

B. Enzyme Contacts with the Inhibitor/Substrate

A large number of crystal structures of the HIV-1 protease complexed to different peptidomimetic inhibitors have provided a wealth of detail concerning binding interactions.[33,62] All of these inhibitors bind to the protease in a similar fashion. The main-chain torsion angles of all inhibitors (and probably all substrates) correspond to an extended conformation, like that of inhibitor U-75875 in Figure 3, A and B, the only exception being the torsion angles between the P_1 and P_1' amino acids. Gustchina et al.[62] have analyzed the hydrogen bond interactions between 15 peptide-based inhibitor main-chain amides and carbonyl oxygens and atoms in the HIV-1 protease. These investigators found that these contacts are very similar for all of the complexes and include a number of hydrogen bonds between the inhibitor main chain and the two flaps, in one direction, and the floor of the catalytic site, in the other (Figure 4). One can visualize the enzyme dimer:inhibitor interaction as a three-strand β-sheet with the inhibitor as the central strand. The nonhydrolyzable scissile bond replacement of the hydroxyethylene transition-state insert of each inhibitor is aligned with the catalytic carboxylates of Asp_{25} and $Asp_{25'}$. The oxygen of the insert OH group is positioned within hydrogen-bond distance of these carboxylates. In the case of the dihydroxyethylene isostere insert (e.g., U-75875,
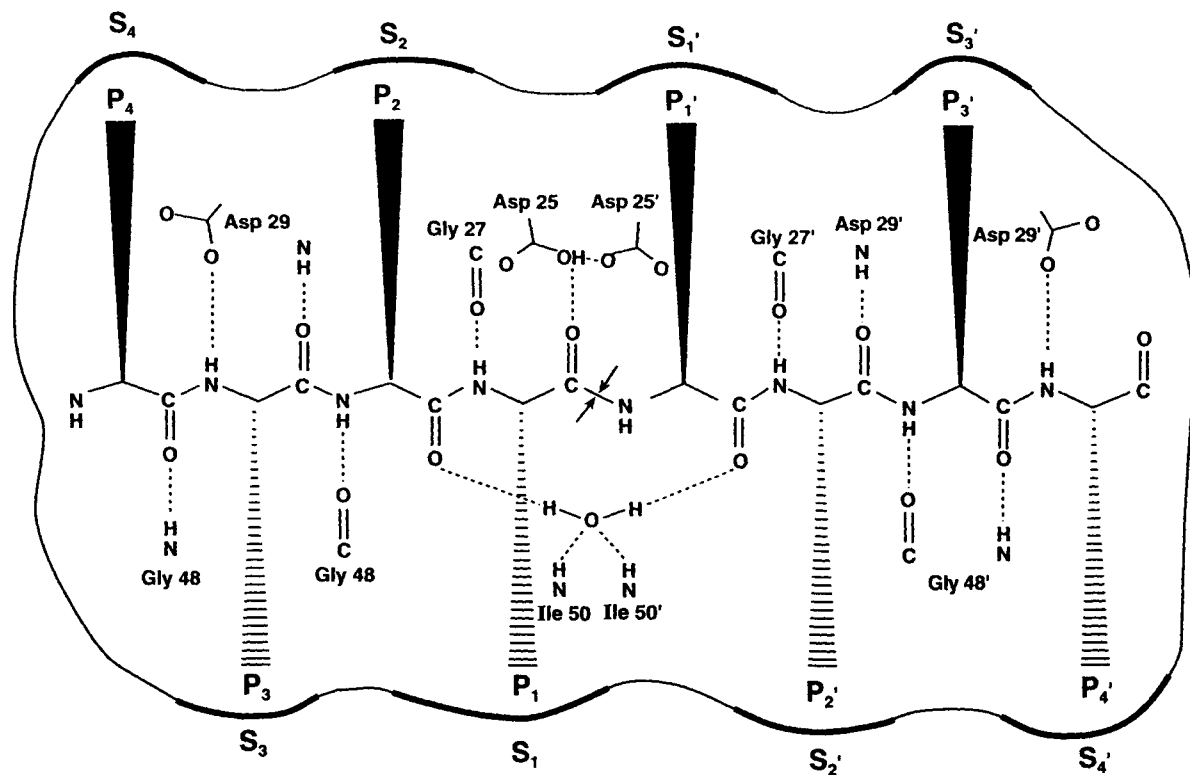
Figure 4. Active site contacts between HIV-1 protease and ligand. Patterns are shown of hydrogen bonds between the enzyme residues at the active site and the backbone of a generic peptide.

Figure 3, A, B, and C) one of the hydroxyl groups of the dihydroxyethylene isostere is positioned such that it forms hydrogen bonds with both of the two catalytically essential aspartic acid residues, whereas the other hydroxyl group interacts with only one of the two aspartates. Calculations suggest that the inhibitor backbone:protein interactions (which are not sequence-specific) contribute 56–68% of the total interaction energy.[62] This might explain the broad substrate specificity of the protease in that differences in substrate binding and catalysis cannot be explained solely on the basis of independent interactions of particular types of amino acid side chains at each position in a peptide ligand. However, the authors also point out that the interactions of ligand side chains in the protease subsites are critical for the specific binding. This would tend to support the idea of a cumulative specificity working in concert with a sequence-independent substrate backbone interaction with the protease. In any case, there is little doubt that substrate recognition and catalysis are strongly sequence dependent. Further analysis[62] indicated that protease inhibitors form tighter hydrogen bonds with the flexible flaps and the bound water molecule than with the floor of the catalytic site. These considerations will be explored further in a later section dealing with inhibitor design by workers at Dupont-Merck.

Additional information on the contacts between enzyme and inhibitor is found in the review of Wlodawer and Erickson.[33] These investigators analyzed the subsites that interact with the corresponding P-site residues in nine peptide-based inhibitors (Table 1). The catalytic cleft is rather hydrophobic: 16 of 30 unique subsites are hydrophobic, six are glycine residues, and the remaining eight residues are charged ($Asp_{25/25'}$, $Asp_{29/29'}$, $Asp_{30/30'}$, and $Arg_{8/8'}$). Analysis of subsite compositions (Table 1) shows that nearly every amino acid that contacts an inhibitor does so at more than one subsite position. Furthermore, the strength of binding is not a function of either the total number of contacts or the total number of subsite interactions. This is exemplified by the fact that the inhibitor MVT-101 (Figure 5, top), with 175 contacts, is relatively weakly binding (k_i = 780 nM), whereas the potent Ro-31-8588 with a K_i of <1 nM (Figure 5, bottom) makes the fewest number
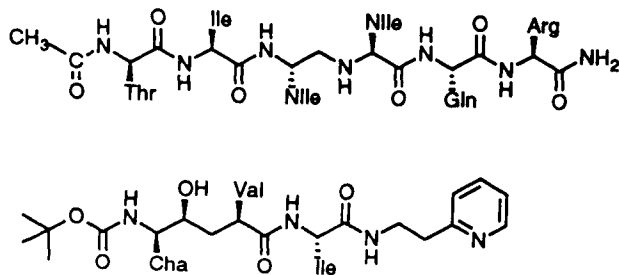
Figure 5. Chemical structures of MVT-101 (top) and Ro-31-8588 (bottom).

Table 1. Subsite Compositions for HIV PR: Frequency of Occurrence of a Given Residue Making Inhibitor Contacts in a Particular Subsite

Residues from Subunit 1

Subsite	N^a	R8	L23	D25	G27	A28	D29	D30	V32	I47	G48	G49	I50	P81	V82	I84	(M46)	(F53)
S5	1																1	1
S4	2						1	2		2	2						1	1
S3	6				5	6	6	2		2	6	1					1	1
S2	9			2	6	8	6	8	6	7	9	8	1			5		
S1	9			9	9	5				1		7	8					
S1'	9	2	6	9	1								3	5	7	8		
S2'	9	1											8					
S3'	7	6												2	4			

Residues from Subunit 2

Subsite	N^a	R8'	L23'	D25'	G27'	A28'	D29'	D30'	V32'	I47'	G48'	G49'	I50'	P81'	V82'	I84'	K45'
S5	1																
S4	2																
S3	6	4												1	1		
S2	9	1											7				
S1	9		7	9	4									8	8	7	
S1'	9			2	9	1					5	9	6				
S2'	9				8	8	8	4	6	5	9	1				4	
S3'	7				2	1	7	3		4	7						1

Notes: $^a N$ represents the number of complexes that contributed to a particular subsite analysis.

Reproduced with permission from Wlodawer and Erickson.[33]

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of contacts with the enzyme. Kinetic and crystallographic studies with hydroxyethylene isostere inhibitors[63] showed that a "core" structure for tight binding consists of four side chains corresponding to the substrate residues P_2–P_2'. It follows that truncation of protease inhibitors may not lead to significant loss of potency. In fact, truncation may lead to increased potency as long as P_2 through P_2' components are left intact. Addition of more groups can actually interfere with an otherwise optimal binding of the P_2/P_2' core structure of the inhibitor with groups in the enzyme. Such considerations have been of importance in de novo design of inhibitors.

C. Structural Comparisons Among HIV-1, HIV-2, and SIV Proteases

Although the HIV-1 protease has dominated research, HIV-2 and SIV proteases have also been investigated to some extent. The HIV-2 and SIV proteases are important because (1) HIV-2 is a disease prominent in Africa, with several cases reported in the Western countries, and with the potential to spread further; (2) SIV is a virus that affects certain primates and causes an AIDS-like disease; it may, therefore, provide an animal model (e.g., see case for U-75875 later); and (3) various HIV-1 isolates, selected in vitro on the basis of their resistance to HIV-1 protease inhibitors, carry mutated amino acids in the protease sequence that are naturally occurring in the HIV-2 and SIV protease, and these observations may be exploited in drug design.

```
        1                                               33
HIV-1       I T    Q    L    I K  G    L K   A                T V L
HIV-2   P Q F S L W  K R P V V T A Y I  E G Q P V E V L L D T G A D D S I V
SIV                 R              H


        34                                              66
HIV-1   E E M S   P G R W K    M I              K V R Q   D Q I L    I
HIV-2   A G I  E L G N N Y  S P K I  V G G I  G G F I  N T K E Y K N V E I  E V
SIV     T          P H    T


        67                                              99
HIV-1   C G H   A I G   V  L V   P    V    I      L     Q I   C T     F
HIV-2   L N K K V R A T I   M T G D T P I  N I  F G R N I  L T A L G M S L N L
SIV       G   R I K G                            L
```

Figure 6. Comparison of the amino acid sequences of HIV-1, HIV-2, and SIV proteases (Tomaselli et al.[34]). The complete sequence of HIV-2 is shown; the amino acid changes occurring in the HIV-1 and SIV are indicated in their respective rows.
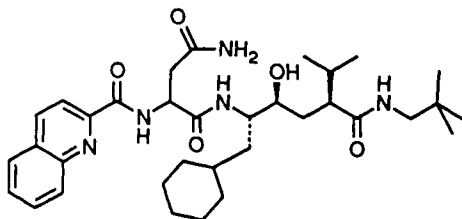
Figure 7. Chemical structure of U-92163.

Both HIV-2 and SIV proteases have been cloned and expressed in bacteria[20,64-67] and have been chemically synthesized.[31,68] Simple purification schemes have also been reported.[20,22] A comparison of the amino acid sequences of HIV-2 and SIV proteases (Figure 6) shows that the two enzymes are 90% identical, and if conservative substitutions are considered, the degree of identity is near 100%. Therefore it is not surprising that the two enzymes are very close in their specificity for viral (Scheme 1) and nonviral substrates and show similar inhibition profiles toward peptidomimetic inhibitors.[68]

The 3D structure of HIV-2 protease complexed to the peptidomimetic inhibitor U-75875 (dihydroxyethylene insert, Figure 4) and U-92163 (hydroxyethylene insert, Figure 7) have been reported by Mulichak et al.[65] Rose et al.[66] determined the 3D structure of SIV protease covalently bound to the inhibitor 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP), and Zhao et al.[67] determined the 3D structure of SIV protease bound to the hydroxyethylene isostere inhibitor SKF-107457 (Figure 8). In the latter paper it is reported that the structures of the SIV and HIV-1 proteases complexed to the same inhibitor are very similar yet vary significantly in three surface loops (not part of the active site cavity) composed of amino acids 15–20, 34–45, and 67–70. The other two reports on SIV and HIV-2 proteases reached similar conclusions. Furthermore, Zhao et al. (1993)[67] pointed out that the largest difference in protein backbone atoms is in the 34–45 loop, where the α-carbon of residue 40 differs by as much as 4.1 Å. This loop is next to the flap and may be responsible for the reorientation of the tips of the flaps. Such a structural change
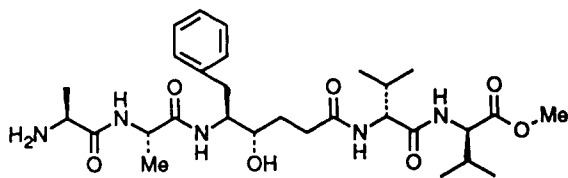
Figure 8. Chemical structure of SKF107457.

could contribute to the previously observed differential binding affinity reported for certain inhibitors[69–71] and different specificity for certain substrates[69,72,73] reported for HIV-2/SIV and HIV-1 proteases. Although the 3D structures of SIV and HIV-2 proteases have not been compared directly, their individual comparison to the HIV-1 protease strongly suggests that they are essentially identical and can be interchanged in kinetic, inhibition, and structural studies. Moreover, the residues that form the active site cavity in the HIV-1 protease (8, 23, 25, 27–30, 32, 47–50, 53, 80–82, and 84) are identical in SIV and HIV-2 proteases. The following variations have been observed from HIV-1 to HIV-2 proteases: V32I, I47V, and V82I. Residues 32(32′) and 47(47′), which form part of the subsite S2 (S2′), are positioned across from one another in the binding cleft, and complementary substitutions at these two positions change, only slightly, the shape but not the size of the cleft.[65] However, substitution of $V_{82/82'}$, which is part of the S1(S1′) subsite, with the bulkier Ile in HIV-2/SIV protease, results in the narrowing of that subsite.[65] When structures of complexes of U-75875 with both HIV-1 and HIV-2 proteases were compared,[65] the most dramatic difference in the binding cleft is a 1.5-Å main-chain deviation of residues 80–83, which are shifted to allow more room for the bulky P_1 CHA side chain in the HIV-1 complex. This shift in backbone conformation may explain in part the difference in K_i values between HIV-1 protease ($K_i < 1$ nM) and HIV-2 protease ($K_i = 30$ nM) on binding U-75875. Other inhibitors containing CHA or Leu in P1 showed K_i values as much as two or three orders of magnitude higher for the HIV-2/SIV proteases than for the HIV-1 enzyme.[69,70] Observations in line with those made for U75875 were reported[67] with SIV protease complexed to SKF-107457. The side chains of this inhibitor at P_2, P_1, P_1', and P_2' are methyl, benzyl, hydrogen, and isopropyl, respectively. SKF-107457 binds with similar K_i to both HIV-1 and SIV proteases. On the other hand, if a bulky benzyl group is added at the P_1' (replacing a hydrogen) of the inhibitor, it binds SIV protease with a K_i three times higher than HIV-1 protease. A similar line of reasoning can be used to support observations, mostly from our laboratory,[69] that HIV-2 and SIV proteases are better able to hydrolyze substrates with small amino acids in the P_1 and P_1' positions. As mentioned above, factors such as differences in the loop 35–45 and chain flexibility may also contribute to the differences in specificity and inhibition between HIV-2/SIV and HIV-1 proteases. Based upon the differential behavior of HIV-1 and HIV-2/SIV proteases toward certain substrates and inhibitors, Zhao et al.[67] suggested that to design inhibitors that are equally effective against all three proteases, it would be important to balance the size of the side chains at P_2, P_1, P_1', and P_2' to ensure a proper fit of the inhibitor in a shallower active site. This should also be taken into consideration when designing inhibitors toward HIV-1 protease mutants which carry altered residues that are naturally occurring in the SIV and HIV-2 proteases.

IV. THE SEARCH FOR INHIBITORS:
THE RENIN EXPERIENCE

As mentioned earlier, the focus of this chapter is on HIV-1 protease inhibitors with potential applications as drugs against AIDS. Although it might be obvious, it is important to stress at the outset that the job of making an enzyme inhibitor for research purposes is an entirely different undertaking from that of designing an inhibitor as a therapeutic agent. A protease inhibitor drug must not only show strong binding to the target protease (picomolar K_i range), but it must be effective and preferably nontoxic in blocking viral maturation in an infected person. Ideally, the drug will be active against a wide spectrum of viral isolates and thus be invulnerable to strains that might otherwise mount resistance, and it should be synergistic with drugs against other viral targets. Blood and/or intracellular concentrations of the drug need to reach and maintain, at least for several hours, levels far above the K_i value for the target. Oral bioavailability is highly desirable but often difficult to achieve; this is also true for a long half-life. Since the virus has access to the brain, the protease inhibitor should be able to cross the blood–brain barrier. As if this were not enough to ask, the drug should be cheap to make and affordable to AIDS patients around the world. It is not surprising that few inhibitors of the HIV-1 protease make it to clinical trials, and thus far, none are on the market.

By the time the HIV-1 protease had been recognized as a therapeutic target in AIDS, a number of pharmaceutical companies had already invested years of research in trying to develop inhibitors of the related aspartyl protease renin.[74] In addition to the wealth of experience gained in design of these renin inhibitors, there existed stockpiles of substrate-based peptidomimetic renin inhibitors containing noncleavable inserts as replacements for the scissile-bond amino acid partners. The

Xaa ψ (CH₂NH) Yaa
reduced bond isostere

Xaa ψ (CH[OH]CH₂) Yaa
hydroxyethylene isostere

Xaa ψ (CH[OH]CH[OH]) Yaa
dihydroxyethylene isostere

Xaa ψ (CH[OH]CH₂N) Yaa
hydroxyethylamine isostere
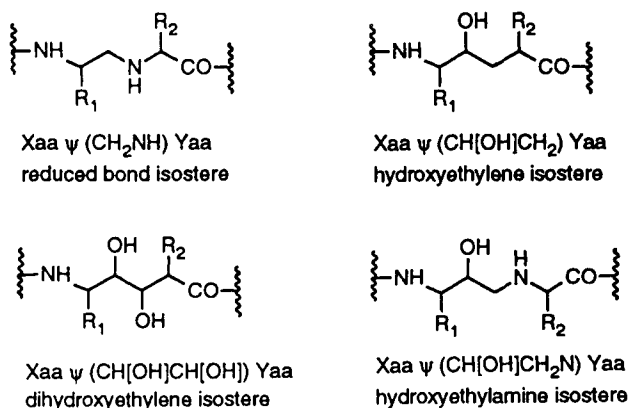
Figure 9. Selected $P_1–P_1'$ dipeptidemimetic replacements of amino acid residues at the cleavage site.

chemistry of these inserts, transition-state mimics of amide hydrolysis, had been explored intensively. These inserts include reduced bond (CH_2NH), hydroxyethylene ($CH[OH]CH_2$), dihydroxyethylene ($CH[OH]CH[OH]$), and hydroxyethylamine ($CH[OH]CH_2N$) isosteres[74,75] (Figure 9).

A. The Upjohn Inhibitor: U-75875

Early examples of "hits" from the renin inhibitor inventory at Upjohn include the hydroxyethylene isostere-containing compound U-71038 (ditekiren, Boc-Pro-Phe-N-MeHis-Leuψ[CH(OH)CH$_2$]Val-Ile-Amp, K_i = 70 pM against purified cloned human renin), which was shown to be a good inhibitor of HIV-1 protease, with a K_i value of 10 nM.[69] However, it showed only weak antiviral activity in HIV-infected cells (Table 2). Truncation of the molecule to a peptide with a phenoxyacetyl blocking group yielded a small inhibitor, U-71017, with a comparable K_i value but with improved activity in cell assays (Table 2). Further enhancement of the antiviral activity was observed in U-79213 by replacing Leu with cyclohexylalanine (CHA; Table 2). Good potency was still observed when this inhibitor was shortened by replacing the POA-His moiety with a *tert*-butylacetyl (TBA) group to obtain the relatively small compound U-81749E[76] (Table 2). U-81749E, however, showed negligible renin inhibition.[76] Screening renin inhibitors containing a diol insert (dihydroxyethylene) against the HIV-1 protease also identified some inhibitors with good activity, and this led to the design and synthesis of additional compounds with diol inserts.[70,77] As an example of the power of a diol insert, substitution of the hydroxyethylene moiety of U-79213 with a dihydroxyethylene isostere resulted in a compound, U-88443, with an improved K_i value and higher antiviral potency (Table 2). Moreover, replacement of the POA moiety with a NOA group at the N-terminus resulted in U-75875 (IC_{50} = 2 nM against human plasma renin), which exhibited a K_i value of <1 nM against the HIV-1

Table 2. Structure–Activity Analysis of Substrate-Based Inhibitors of HIV-1 Protease with the Hydroxyethylene (Upper Panel) and Dihydroxyethylene (Lower Panel) Inserts at the P$_1$-P$_1$' Sites

Compound	Structure	K_i, nM	% inhibition[a] p24 Synthesis in HIV-1 PBL
U-71038	Boc-Pro-Phe-$^\alpha$MeHis-Leuψ[CH(OH)CH$_2$]Val-Ile-Amp	10	28%
U-71017	POA-His-Leuψ[CH(OH)CH$_2$]Val-Ile-Amp	10	76%
U-79213	POA-His-CHAψ[CH(OH)CH$_2$]Val-Ile-Amp	10	90%
U-81749E	TBA-CHAψ[CH(OH)CH$_2$]Val-Ile-Amp	70	70%
U-88443	POA-His-CHAψ[CH(OH)CH(OH)]Val-Ile-Amp	2	100%
U-75875	NOA-His-CHAψ[CH(OH)CH(OH)]Val-Ile-Amp	<1	100%

Note: ^{a}Measured at 1 μM test compound.

protease (Table 2) and served as an active site titrant of the enzyme.[77] U-75875 showed potent antiviral activity (ED_{50} = 3 nM) against HIV-1 infected human peripheral blood lymphocytes.[10] It irreversibly prevented *gag* processing in particles produced by human T cells chronically infected with HIV-1, and the viral particles, released in the presence of this inhibitor, had an immature appearance and were noninfectious. U-75875 was shown to be effective against the three related lentiviruses: HIV-1, HIV-2, and SIV_{mac}. In spreading infection experiments carried out over a period of 1 month, U-75875 completely blocked the spread of infection in primary cell lines as well as in continuous cell lines.[10] U-75875 was also very effective in reducing the virus titer in chronically infected macrophages, an important in vivo reservoir of HIV.[78]

In SIV-infected PBMC, U-75875 was shown to inhibit p26 antigen as well as production of infectious SIV. This finding in vitro offered the opportunity to study the effect in vivo of an HIV protease inhibitor on SIV infection in monkeys. Rhesus monkeys were treated with U-75875 at doses of 7 or 20 mg/kg/day for 4 weeks by continuous intravenous infusion, beginning 6 hours prior to intravenous inoculation with SIV/Delta B670.[79] The treated animals demonstrated a delayed onset of antigenemia, delayed attainment of peak antigenemia, and shortened duration of antigenemia compared to vehicle-treated controls. The spread of the infection as determined by DNA-PCR analysis from blood mononuclear cells was decreased and the level of proviral burden was also decreased. The titer of infectious virus in serum was also decreased in a dose-dependent manner. This appears to be the only report on efficacy of a protease inhibitor in vivo in this relevant monkey model and lends support to the notion that HIV protease inhibitors are worthwhile as potentially effective anti-HIV agents in humans. It should also be noted that U-75875 is at least 100-fold less active toward inhibition of the SIV protease as compared to the HIV-1 enzyme,[68] so this observation of efficacy in the monkey is all the more remarkable.

B. The Roche Inhibitor Ro 31-8959 (Saquinavir): Development Leading to Clinical Trials

An uncommon feature of the HIV proteases is that, in contrast to general mammalian endopeptidases, they are able to cleave a number of substrates containing proline at the P_1' site. This somewhat unique aspect of the HIV-1 protease specificity was generally recognized as a basis for the rational design of selective inhibitors for this viral enzyme. The hydroxyethylamine transition-state mimetic was readily adapted to the P_1' proline containing substrate sequences, and active inhibitors were prepared.[80] Extensive structure–activity studies of the Roche series revealed that marked improvement in potency was achieved when the imino acid proline at the P_1' site was replaced by the (*S,S,S*)-decahydro-isoquinoline-3-carbonyl group. The resulting compound, Saquinavir (Figure 10), showed very high binding affinity to both HIV-1 and HIV-2 proteases, with K_i values below 1 nM. In another
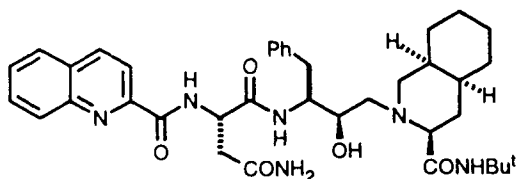
Figure 10. Chemical structure of Ro-31-8959.

study of inhibitors with P_1 Pro, Rich et al.[81] reported that the hydroxyethylamine containing inhibitor JG-365 (Ac-Ser-Leu-Asn-Pheψ[CH(OH)CH$_2$N]Pro-Ile-Val-OMe, $K_i = 0.66$ nM; Figure 11) showed a preference for the S isomer at the hydroxyl group of the hydroxyethylamine moiety, as suggested by the crystal structure of the complex with HIV-1 protease.[82] Subsequent synthesis of the specific diastereomers from chiral precursors confirmed the strong preference for the S stereocenter at the hydroxyl group.[83] In sharp contrast, Saquinavir, with the (S,S,S)-decahydro-isoquinoline-3-carbonyl group, showed clear preference for the R stereoisomer at the hydroxyl group of the hydroxyethylamine moiety. This was confirmed by crystallographic analysis of the Saquinavir/HIV-1 protease complex.[84] The different preferences of the stereoisomer at the hydroxyl groups in these two compounds reflected different binding modes, which appeared to be related to both the overall length of the inhibitors and the different amino acid residues at the P_1' site (proline versus the (S,S,S)-decahydro-isoquinoline-3-carbonyl group).

Saquinavir is a highly selective inhibitor for HIV proteases. It gives a K_i value of 0.12 nM against the HIV-1 protease at pH 5.5, while showing less than 50% inhibition of human aspartic proteases (renin, pepsin, gastricsin, cathepsin D, and cathepsin E) at a concentration of 10 μM, and no inhibition of representative proteases from the serine, cysteine, and metallo classes.[85] It also showed potent antiviral activity. For example, in JM cells infected with the HIV-1 strain GB8, the mean ED_{50} value was 2.5 nM, and inhibition of p24 production in CEM cells chronically infected with HIV-1 strain IIIB was demonstrated at inhibitor concentrations as low as 1 nM. Saquinavir was shown to be synergistic with AZT or ddC as a two-way or a three-way combination in CEM-T4 cells infected with HIV-1 strain GB8.[86,87] Moreover, in HIV-1-infected PBMC, additive to synergistic effects with AZT, ddC, or recombinant interferon-αA were reported.[88] Combinations of
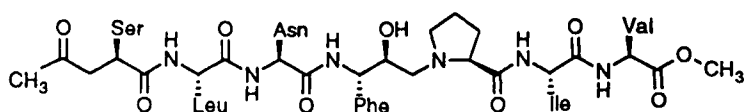
Figure 11. Chemical structure of JG-365.

Saquinavir, AZT, and interferon-α were also studied in adherent monocyte cultures infected with HIV-1 Ba-L, a monocytotropic strain of HIV.[89]

Saquinavir was the first HIV protease inhibitor to enter clinical trials, and these began in early 1991. Although oral bioavailability was low, the mean plasma concentration at 600 mg t.i.d. was reported to be 70 nM, which is higher than the in vitro ED_{90} value. The oral bioavailability increased markedly by administration of the drug with food. A 16-week study in HIV-infected patients (CD4 counts of 50–250) showed that the drug was well tolerated and there was a trend toward an increase in CD4 counts and a decrease in p24 levels, with maximal effect at 4 weeks.[90] With a combination of 600 mg t.i.d. of Saquinavir and 200 mg t.i.d. of AZT, there was a trend toward increasing CD4 to a greater extent than with either monotherapy. Saquinavir is currently in phase III clinical trials. Finally, the U.S. National Institute of Allergy and Infectious Diseases AIDS Clinical Trials Group has conducted a study with a combination of Saquinavir, the nucleoside analogs zalcitabine (Roche's Hivid), and AZT. The study, ACTG-229, enrolled 302 patients who had CD4 cell counts ranging from 59 to 300/mm and who had received AZT therapy for at least 4 months before enrollment. The patients were randomized either to the triple combination or to AZT combined with one of the other two drugs. The dosages used were Saquinavir, 600 mg t.i.d.; Hivid, 0.75 mg t.i.d.; and open label AZT, 200 mg t.i.d. The study lasted 24 weeks. The triple combination was as safe as and more effective in the treatment of HIV infection than the dual combinations of Saquinavir and AZT, or Hivid and AZT. The general trend of all three groups was a rise in CD4 cell counts during the first 8 weeks of therapy, followed by a gradual decline. The triple combination group registered the greatest increase in CD4 cell counts. At 24 weeks, 31% of the patients in triple combination had CD4 cell counts that had returned to baseline, compared with 37% on Saquinavir/AZT and 55% on Hivid/zidovudine. Reduction in viral burden was greater with the triple combination over the 24-week study. It would be interesting to see whether a triple combination of AZT, Saquinavir, and a non-nucleoside RT inhibitor may provide a more lasting benefit.

C. The Searle Compound: SC-52151

Application of the hydroxyethylurea isostere, previously incorporated into renin inhibitors, to the development of HIV-1 protease inhibitors resulted in the potent inhibitor SC-52151[91] (Figure 12). This urea isostere-containing inhibitor closely resembles Saquinavir in structure, and it shows marked preference for the *R* stereoisomer at the hydroxyl group of the hydroxyethylurea moiety. The crystal structure of an HIV-1 protease complex with an analog of SC-52151 containing an *n*-butyl group in place of the C-terminal *tert*-butyl group reveals the terminal *n*-butyl group in the S_1' subsite and the isobutyl group in the S_2' subsite. This binding mode was unexpected, based upon a previously reported structure of a urea-containing renin inhibitor bound to endothiapepsin.[92] SC-52151 inhibits
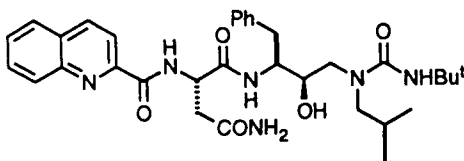
Figure 12. Chemical structure of SC-52151.

HIV-1 protease with an IC_{50} value of 6 nM and shows no significant activity at a concentration of 10 µM against human renin, porcine pepsin, or bovine cathepsin D. In CEM cells infected with strain $HTLV_{IIIB}$, it showed an ED_{50} value of 21 nM. In human PBMC, it was also found to be equally effective against fresh clinical isolates, including AZT-resistant strains.[93]

In a phase I clinical trial, SC-52151 was given orally to HIV-infected asymptomatic volunteers at single escalating doses. No adverse reactions were noted; peak plasma concentrations occurred at 1.5 hours, and plasma levels declined with a half-life of 2 hours. At doses of 100, 250, 500, and 1000 mg, C_{max} values were 61, 144, 294, and 827 ng/ml, respectively. It was projected that 600 mg t.i.d. would result in a C_{max} of 0.35 µM and a C_{min} of 0.01 µM, the ED_{50} value in vitro.[94]

D. The KNI Series: KNI-272

Kiso and co-workers[95–97] also considered the unique ability of the HIV-1 protease to cleave the Phe-Pro bond, in this case in the heptapeptide: H_2N-Ser-Phe-Asn-Phe-↓-Pro-Ile-Val-NH_2. This peptide represents the natural processing sites TF/Pr and p17/p24 in the viral polyprotein (Schemes 1 and 2). Based upon the transition state isostere concept, these workers designed and synthesized a novel class of HIV-1 protease inhibitors containing allophenylnorstatine (APNS; (2S,3S)-3-amino-2-hydroxy-4-phenylbutyric acid) as a transition state mimic (Scheme 2).

Having proved that the *syn* diastereomer H_2N-Ser-Phe-Asn-Apsn-Pro-Ile-Val-NH_2 (KNI-93; IC_{50} = 5 nM) was more active against the HIV-1 protease than the *anti* compound with phenylnorstatine (PNS) in place of APNS (KNI-122; IC_{50} = 100 nM), they embarked upon extensive SAR studies based upon truncation and modification of KNI-93 (Scheme 2 and Table 3). An interesting lead was found by substituting the N-terminal Ser-Phe with a benzyloxycarbonyl (Z) group, and the C-terminal Ile-Val with -NHBu'. These modifications gave KNI-102, an inhibitor that bound less tightly to the protease (IC_{50} = 89 nM) but which showed improved antiviral activity over its parent KNI-93 (Table 3). KNI-102 was further optimized by replacing Z with 1-naphthyloxyacetyl (NOA) and Pro with L-5,5-dimethylthiazolidine-4-carboxylic acid (DMT). This gave a potent and selective inhibitor of the HIV-1 protease NOA-Asn-APNS-DMT-NHBu' (KNI-174; Scheme 2) with an IC_{50} of 2.8 nM and a K_i of 6.8 pM (Table 3). KNI-174 showed about 5% bioavailability
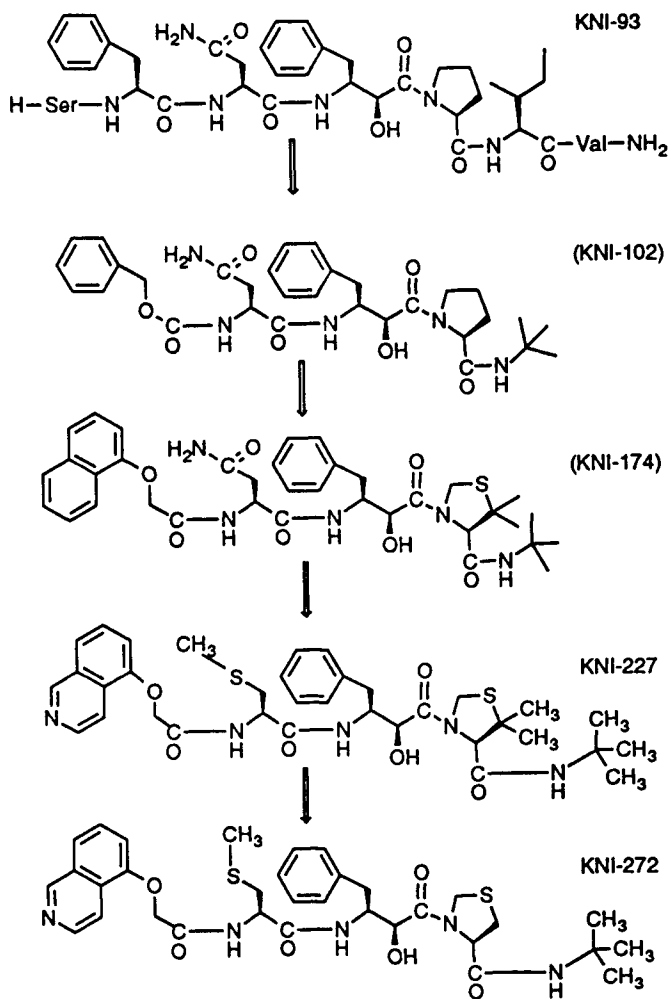
Scheme 2. The development of inhibitor KNI-272. Data on HIV protease inhibition and antiviral activity of the KNI compounds are given in Table 3.

after i.d. administration in rats, and an IC_{50} of 0.4 µM against HIV-1 LA1 in ATH8 cells.

To improve bioavailability and potency by increasing penetration through cell membranes, several other changes were made. The 5-isoquinolinyl oxyacetyl (iQOA) was incorporated at P_3 and methylthioalanine (MTA) at P_2 to give iQOA-MTA-APNS-DMT-NH-Bu' (KNI-227; Scheme 2). A final analog, KNI-272, was made by replacing the DMT group with L-thiazolidine-4-carboxylic acid (THZ) to give iQOA-MTA-APNS-THZ-NH-Bu' (Scheme 2). Both of these inhibitors were

Table 3. KNI Inhibitors Series: HIV-1 Protease Inhibition and Antiviral Activities Against HIV-1 LAI in ATH8 Cells

Compounds	K_i $(nM)^a$	IC_{50} $(nM)^b$	IC_{50} $(\mu M)^c$	TC_{50} $(\mu M)^d$	TC_{50}/IC_{50}
...SQNY*PIV...^{e}	—	—	—	—	—
...SFNF*PQI...^{f}	—	—	—	—	—
[SFNF*PIV-NH$_2$]^g	—	—	—	—	—
KNI-93	NR	5	poor	NR	NR
KNI-102	NR	89	1.1	>20	>18
KNI-174	0.0068	2.8	0.4	30	75
KNI-227	0.0023	2.3	0.1	40	400
KNI-272	0.0055	6.5	0.1	>50	>500

Notes: ^{a}HIV-1 protease inhibitor constant determined using synthetic C67A/C95A protease.

^{b}Concentration of inhibitor at which 50% of HIV-1 protease activity is inhibited.

^{c}50% inhibitory concentration against cytopathic effect of HIV-1.

^{d}50% toxic concentration of the compound.

^{e}p17/p24 region (see Scheme 1).

^{f}TF/PR region (see Scheme 1).

^{g}Peptide prepared after p17/p24 and TF/PR regions; NR, not reported.

The chemical structures of the KNI compounds are given in Scheme 3.

very potent: KNI-227 had an IC_{50} of 2.3 nM and a K_i of 2.3 pM against the HIV-1 protease and showed much improved antiviral activity at $IC_{50} = 100$ nM. Similar activity was seen in KNI-272 (Table 3), but this inhibitor showed higher oral bioavailability than KNI-227, i.e., 42.3% versus 5%, respectively (i.d. in rats).

It should be noted that the chirality of the APNS OH group is a crucial factor for inhibitor potency. Mimoto et al.[98] have shown that the K_i value of the (S) isomer of KNI-272 is 20-fold lower than that of the (R) isomer. Baldwin et al.[99] determined the 3D structure of HIV-1 protease complexed to the (S) isomer of KNI-272. Modeling of the (R) isomer into the active site cleft of this structure led them to conclude that the increase in K_i value is due to loss of optimal interaction with the active-site aspartic acid residues.

The experience with the KNI series exemplifies nicely what was stressed at the outset in this section of the chapter. Development of an effective drug is multifactorial; one needs good inhibitory activity toward the target enzyme, potency against the virus, and good pharmacokinetics. These desirable features appear to be present in KNI-272 (Table 3). KNI-227 and -272 were highly selective for the HIV-1 protease, showing negligible activity toward other aspartyl proteases such as renin, pepsin, and cathepsin D. Both compounds were stable to hydrolysis over a period of 24 hours at 37°C in HCl at pH 1 and in the presence of pepsin. KNI-272 combined highly potent antiviral activity with low cytotoxicity ($TC_{50} > 80 \mu M$). The final test for this optimized HIV-1 protease inhibitor will be to see if it blocks viral maturation

in an AIDS patient, and whether, or how easily, the resourceful virus will be able to mount resistance to it.

V. SYMMETRY AS A PRINCIPLE IN INHIBITOR DESIGN

A. The Abbott Series

The striking and unique symmetry of the HIV-1 protease dimer provided a rationale for the design of inhibitors based upon C_2 symmetry. Workers at Abbott were the first to report symmetric series of inhibitors that were selective for the HIV-1 protease. Early in their design work, Abbott scientists considered two things that must be satisfied to exploit the symmetrical enzyme target. The inhibitor had to bind in such a way as to superimpose its symmetry axis over that of the HIV-1 protease, and enzyme subsites that normally interact with asymmetric inhibitors would have to be filled. This line of reasoning led to the design of A-74704 (Figure 13). X-ray crystallographic analysis of the complex between this inhibitor and the HIV-1 enzyme proved that the designed-in binding symmetry had been achieved.[100]

As an extension of their work, J. W. Erickson, D. J. Kempf, and their co-workers at Abbott designed a series of diamino diol symmetric inhibitors in which the C_2 axis bisected the bond connecting the two hydroxyl-bearing carbon atoms.[101] They studied the influence of the stereochemistry of the inhibitors on their mode of enzyme binding. Relative potencies of inhibition by A-77003 and its stereoisomers (Table 4) varied widely, and no uniform dependence was seen with respect to the stereochemistry of the hydroxymethyl group. Furthermore, removal of a single hydroxyl group from the (R,R) diol enhanced inhibition (Table 4). As will be discussed in the following narrative, crystallographic results demonstrate that C_2-symmetric inhibitors may bind in either symmetric or asymmetric modes relative to the positioning of the C_2 axes of the protease and the inhibitor (Figure 14). Main-chain flexibility allows the carbonyl group of Gly_{27} to adjust, depending upon the location of the second diol hydroxyl group. This analysis also underscored the importance of van der Waals interactions within the active site cleft in binding of the inhibitor, in addition to the well-established role of hydrogen bonding to the two active site aspartyl residues.
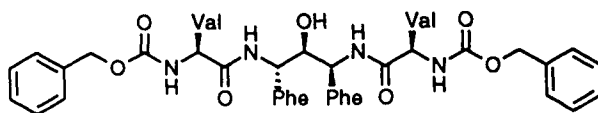
Figure 13. Chemical structure of A-74704.
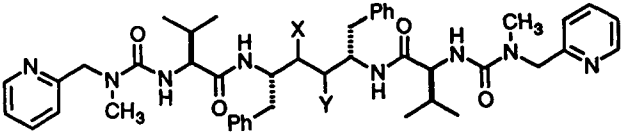
R,S Diol (A-77003 (I) and S Deshydroxydiol A-78791 (IV))

Crystallographic analysis indicates that the R,S diol binds asymmetrically to the HIV-1 protease (Figure 14) and that the bound conformations of I (R,S) and IV (S) (Table 4) are virtually indistinguishable. The structures of the two inhibitors superimpose to within 0.2 Å root mean square (rms) for 54 identical, non-hydrogen atom pairs. The OH group (R) of A-77003 lies centrally between the COOH oxygens of Asp_{25} and $Asp_{25'}$, as does the OH group (S) of A-78791. On the other hand, the OH group (S) of I points away from the active site pocket and seems not to contribute to binding at all. There are indications that the A-77003 OH (S) group increases desolvation energy and entropy loss on binding relative to the deshydroxy analog A-78791; this may explain why IV is a more potent inhibitor than I (Table 4).

R,R Diol (A-76889 (II))

A-76889 binds asymmetrically (Figure 14) to the HIV-1 protease and has an OH group (R) in the same configuration as seen with A-77003. In fact, the conformations of the bound inhibitors are essentially the same (with some differences in the case of the diol due to alteration of the core three torsion angles), and the two have the same number of contacts with the enzyme active site. Yet, II is a tenfold less potent inhibitor of the enzyme than I. The reason for this is that the second OH (R) of the diol (II) induces alteration of the dihedral angles of both this amino acid and the R,R diol core, to avoid highly repulsive van der Waals contacts with $Gly_{27'}$. The crystallographic data show that the main chain at $Gly_{27'}$ in II is displaced relative to those in I and IV. It has been suggested that the price to be paid for changing the conformation of $Gly_{27'}$ may be weaker binding of II.

Table 4. Chemical Structure and Biological Activity of C_2
Symmetry-Based Diol Analogs

Compound	X,Y	K_i, soln (pM)	K_i, cryst (pM)	EC_{50} (μM)
A-77003 (I)	R-OH, S-OH	84	12	0.2
A-76889 (II)	R-OH, R-OH	1000	112	1.54
A-76928 (III)	S-OH, S-OH	77	11	0.17
A-78791 (IV)	S-OH, H	35	4	0.16

Note: Reproduced with permission from Hosur et al.[101]

Symmetric

Symmetric
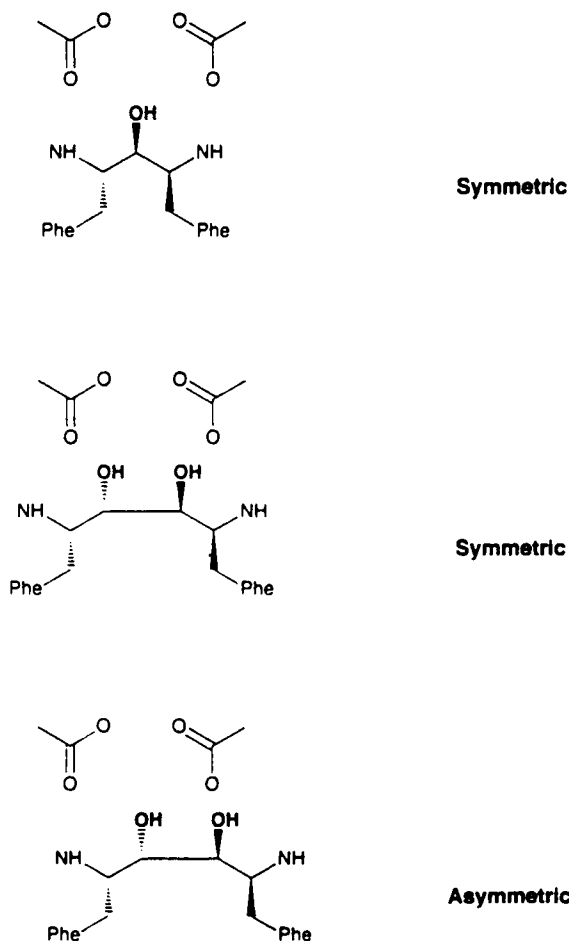
Asymmetric

Figure 14. Symmetric versus asymmetric modes of inhibitor binding to the HIV-1 protease. (**Top**) symmetric mode of binding of the transition state insert exhibited by the pseudo-C_2-diamino alcohol A-74704 (see chemical formula in Figure 12). The C_2 axis of the enzyme relates the two active-site carboxylate groups and nearly passes through the central carbon atom of A-74704 and within 0.2 Å of the hydroxy oxygen. (**Middle** and **bottom**) possible symmetric or asymmetric modes of interaction for symmetry-based diols such as the *S,S* diol A-76928, and the *R,R* diol A-76889, which bind symmetrically and asymmetrically, respectively, to the HIV-1 protease (see details in the text). Ph, phenyl. Reproduced with permission from Hosur et al.[101]

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S,S Diol (A-76928 (III))

A-76928 differs from I only in the sterochemistry of the OH group (S) that interacts strongly with the two catalytic Asp residues. It is nearly as good an inhibitor as I. The S,S diol binds to the HIV-1 protease in such a way that the C-C bond of the isotere is nearly bisected by the enzyme twofold axis (symmetric mode in Figure 14); each OH group makes essentially equivalent interactions with each active site aspartyl residue. Readjustment of the three core torsion angles and a small movement of the enzyme backbone at Gly_{27}, brings the diol core into a tighter fit with the active site.

In all four of these inhibitors, the hydrogen-bonding atoms beyond P_1, P_1' maintain equivalent and symmetric hydrogen bonds in both halves of the inhibitor. The atoms at P_1,P_1' may adjust their positions in an asymmetric way to accommodate the diols within the active site pocket, leading to the observed asymmetric binding mode, while the rest of the atoms are free to obey, and fall in line with, the approximate symmetry of the enzyme. The studies of A-77003 and derivatives show that the ϕ and ψ angles may vary considerably from one inhibitor to another, thus allowing positioning of the inhibitor side chains in the proper enzyme subsites and maximizing hydrogen bonding interactions between the enzyme backbone and the inhibitor. The experience thus far has been that incorporation of C_2 symmetry into the design of a structurally flexible inhibitor, like these derived from peptides, has not necessarily led to inhibitors with greater potency than seen with more conventional compounds, such as U-75875. However, with smaller, structurally constrained inhibitors (see Dupont-Merck DPM323), symmetry is a highly desirable, if not essential feature.

A-77003 gave a K_i of 84 pM and an ED_{50} of 0.2 μM in HIV-infected MT4 cells (Table 4). This compound has an aqueous solubility of 197 μg/ml at pH 7.4 and, after i.v. administration to rats, dogs, and monkeys, its half-lives were shown to be
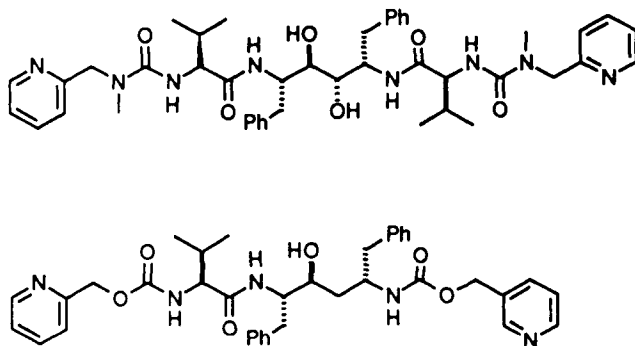
Figure 15. Chemical structures of A-77003 (top) and A-80987 (bottom).

0.5, 1.1, and 3.2 hours, respectively.[102] To improve the oral bioavailability of
A-77003 (Figure 15, top), several changes were made to generate A-80987 (Figure
15, bottom): $K_i = 250$ pM, $ED_{50} = 0.13$ μM in MT4 cells.[103] The oral bioavailability
was now improved to about 13–26% in the three species. Both A-77003 and
A-80987 were brought forward for clinical trials.

In asymptomatic HIV-infected patients (CD4 counts of 200–500), A-77003
(0.35-hour half-life) was given by continuous i.v. administration over 28 days at
0.035 to 0.28 μg/kg/hr doses, showing a C_{max} of 0.6 μg/ml at the highest dose. There
are no serious side effects, although there were a number of reports of local vein
irritation and phlebitis. There was no change in the CD4 or p24 antigen level.[104] In
a phase I clinical trial, A-80987, given orally at 500, 750, and 1000 mg single doses,
resulted in blood levels of 0.5, 2, and 5 μg/ml, respectively.[105]

B. Merck L-735,524: A Hybrid

The discovery of benzocycloalkyl amines as novel C-termini, which resulted
from studies of hydroxyethylene isostere-containing inhibitors, proved to be a key
contribution in the development of HIV-1 protease inhibitors. A small inhibitor,
such as Boc-Pheψ[CHOHCH$_2$]Phe-Ahi with the 1-amino-2-hydroxyindane (Ahi)
C-terminal amide, showed very high enzyme binding affinity[106] ($IC_{50} = 0.3$ nM).
The hydroxyaminopentane amide L-704,486 (Figure 16, bottom) represents a
hybrid that incorporates the decahydro-isoquinoline hydroxyethylamine part of
Saquinavir into the backbone hydroxyethylene isostere with the C-terminal 1-
amino-2-hydroxyindane. The basic amine of the hydroxyethylamine portion was
intended to improve aqueous solubility while maintaining biological activities.
Further substitution of the decahydro-isoquinoline with the pyridylmethyl-piperizine
led to the optimal inhibitor L-735,524[24] (Figure 16, top). L-735,524 inhibits HIV-1
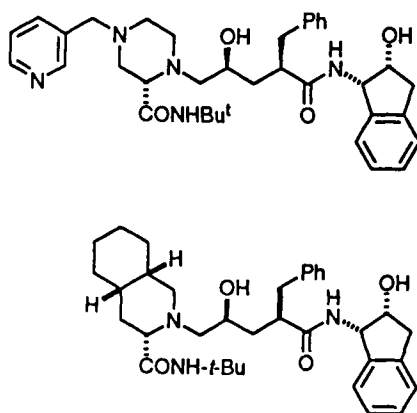
Figure 16. Chemical structures of L-735,524 (**top**) and L-704,486 (**bottom**).

and HIV-2 proteases, with K_i values of 0.34 and 3.3 nM, respectively. In acute infection assay, it is an effective inhibitor of both T lymphoid cell-adapted HIV-1 variants and primary virus isolates ($ED_{95} \leq 100$ nM). It is synergistic with both nucleoside and non-nucleoside RT inhibitors.[24] Despite its peptide character, oral bioavailability in rat, dog, and monkey was determined to be 23, 70, and 14%, respectively. It had a relatively short half-life in plasma ($t_{1/2} = 28$, 34, and 74 min, respectively, after intravenous administrations in the same animals) because of its high clearance rate.

In a phase I clinical trial, the sulfate salt of L-735,524 gave more consistent blood levels than the free base, with higher blood levels in fasted individuals. At 400 mg dose, the C_{max} value was above 1 μM, and blood levels were above 0.1 μM for approximately 6 hours. At 400 mg q.i.d. in HIV-positive patients, the C_{min} value was 0.1–0.2 μM. HIV-infected patients (CD4 counts of 67–665) were dosed for 12 days without significant adverse effects. There was approximately 40% and 70% reduction of p24 on day 2 and day 12, respectively. The p24 gradually rose after drug was discontinued on day 12. That there was no significant change in CD4[107] was disappointing.

VI. HIV-1 PROTEASE AS A PARADIGM FOR DRUG DESIGN

A. The Route to Drug Discovery

The discovery of new drugs is usually a long and difficult process that involves the collaborative efforts of scientists working in various disciplines. The traditional method for drug discovery is to evaluate whether certain synthetic or natural substances (usually a large number) are able to produce a specific effect on the system under study. For example, if the disease is caused by a virus, the first step is to see which available substances are able to block the spread of the virus in infected cell cultures. This serendipitous approach is very long and implies the screening of a large number of compounds to select a few of them, called "leads." The "leads" usually require structural modifications to increase their potency and decrease toxicity. This strategy has been the tried and true stalwart of the pharmaceutical industry; in the usual scenario, one discovers a drug to treat the disease, but the molecular target of the drug remains obscure.

Another complementary and more rigorous approach to drug discovery may be used when the molecular "Achilles' heel" of the disease is known. In the case of AIDS, for example, we know that HIV is the cause; furthermore, we know that if we inhibit the viral reverse transcriptase and/or the protease, viral infection is arrested, at least in cell culture. Rather than perform complicated and dangerous screens using infected cells, assays are based upon purified enzymes and are much easier, cheaper, and safer to carry out. We start with an advantage: we know our molecular targets. Here, too, we will end up with a limited number of "lead" compounds that, after structural modification, will be tested in infected cells.

In spite of the labor involved to obtain them, the majority of the drugs on the market today have been discovered by the two methods just described, and the structural modification of the "leads" was performed by a long, trial-and-error search and refinement process that went on until a reasonable optimization of the product was obtained. Although these traditional routes to drug discovery are still prominent in the industry, they have been supplemented in the past few years by two new and often synergistic approaches: "3D structure-based drug search" and "de novo drug design." Both strategies came from recent technological advances in computational chemistry, and contemporary drug discovery has to be seen in the context of a powerful alliance among a number of disciplines, including x-ray crystallography, molecular modeling, medicinal chemistry, molecular biology, biochemistry, pharmacology, and applied medicine.

3D Structure-Based Drug Search

In the past few years, chemical databases have been converted from a 2D to a 3D format, and 3D search softwares have been developed to find molecules with specific 3D structures from 3D chemical databases. The use of 3D searches in drug development enables the discovery of new compounds with structures similar to those of molecules with known specific biologic activity. A case in point with relevance to the HIV protease is the discovery of haloperidol[108] and derivatives as inhibitors, some of them irreversible,[109] of this enzyme. With this approach, the computational chemist starts out with a collection of active molecules and tries to determine, as accurately as possible, common features such as aromatic rings, hydrophobic groups, and hydrogen-bond sites, and their distribution over the molecule under study. Then a search is made of the database for compounds that satisfy the selected requirements. Some hits may be found and these must be synthesized (if not already available) to identify those that are active and those that are not. The whole process may require several cycles of searching, refinement, and synthesis before producing useful "leads." Most of the programs available zero in on the structure of the inhibitors while ignoring those of the receptors. A different approach is used by a program called DOCK, which requires a high-resolution 3D structure of the receptor under study. It starts out by finding the geometric shape that fits most tightly and shows good van der Waals and electrostatic interaction with the binding site. The hypothetical compound is synthesized and tested for activity. Its structure is then used to screen a 3D database. Compounds discovered with the 3D search approach are being developed in pharmaceutical companies. The great advantage of this approach is that when one gets a hit, the molecule is already available in the inventory, or at least the synthetic methodology is already in hand. And the synthesis of derivatives is easier. In the best case scenario, the "lead" may have already gone to animal trials and passed toxicological and pharmacokinetic tests. However, this technology is of limited use at this time because 3D structures are stored as single conformations (usually the ground state), whereas molecules are flexible, assuming various conformations. Making data-

bases that accommodate conformationally flexible structures is one of the great challenges in this field of research.

De Novo Drug Design

The approach of structure-based design strongly rests on the knowledge of the 3D structure of the macromolecule we want to target, in this case, the HIV-1 protease. As we have already discussed, high-resolution structures of the target in complexation with inhibitors are available. For the HIV-1 protease:inhibitor complexes, the structure with the pattern of electrostatic, hydrophobic, and van der Waals interactions and hydrogen bonds formed by the ligand and the target may be displayed on the computer screen. Otherwise the target alone is displayed and potential ligand structures are generated. The idea is to optimize electrostatic, hydrophobic, and van der Waals interactions and hydrogen bonds between the ligand, whether real or computer-generated, and the target active site. This allows fitting of the ligand to the active site in a "lock and key" fashion. De novo design allows us to create new biologically active molecules from scratch rather than fishing them out of complex databases.

Once the interactions between a candidate compound and the active site are maximized, a few lead compounds are obtained that must be chemically synthesized and tested for their affinity for the target. The inhibition data, together with the crystallographic analysis of enzyme–inhibitor complexes, may reveal why some of the compounds selected by the computer are more active than others. After several cycles of modeling, chemical synthesis, inhibition studies (K_i determination), and structural analysis, a few compounds with high affinity and high specificity for the enzyme will be identified. This is a crucial point in drug discovery no matter what approach was used to reach it. In the case of HIV-1 protease inhibitors, the compounds have to be tested in infected cells and in animal models to see if prerequisites such as potency, specificity, bioavailability, toxicity, good pharmacokinetics, and lack of resistance by viral strains are met. Any serious deficiencies in these areas must be dealt with before going to the clinics; i.e., the iterative cycle that includes remodeling, retesting, etc. could go on several times before the drug enters human trials.

A major problem with antiviral compounds is the selection of viral resistance to the drug, and this has been shown to be especially pertinent to HIV and related viruses that show exceptionally high mutation rates. Obviously it is important to understand the structural basis for drug resistance. Usually some structural features of the target have changed. Molecular biology and biochemistry will indicate alterations in specific amino acids; furthermore, they will provide the mutant enzyme that can be crystallized in complex with the drug. Crystallography will tell us which interactions between the drug and the enzyme have been compromised. Computer modeling will suggest a new compound, and synthetic chemistry will allow us to synthesize it. Inhibition studies will tell us if the suggestion is acceptable. Finally, the in vitro and in vivo studies with the real virus are the ultimate

judges as to the effectiveness of the product. Some examples of this whole process described above will be given in the following section.

B. Screening for Lead Templates—SAR for Refinement: U-96988

The example of U-96988 combines a serendipitous approach to the discovery of a nonpeptide HIV-1 protease inhibitor template, whereupon rigorous structural analysis led, ultimately, to design of a different series of inhibitors. From a high-volume, broad screening of the Upjohn compound collection for HIV protease inhibitory activity, 4-hydroxycoumarin (warfarin) (Scheme 3) was identified as a weak inhibitor, with an IC_{50} value of approximately 30 µM. Additional compounds with similar structures were then tested as potential inhibitors, and another 4-hydroxycoumarin, phenprocoumon, was discovered as a competitive inhibitor, with a K_i value of approximately 1 µM against both HIV-1 and HIV-2 proteases.[110] In HIV-1-infected PBMC, phenprocoumon showed very weak antiviral activity, with an ED_{50} value of 1–3 mM. A crystal structure of the phenprocoumon/HIV-1 protease complex confirmed the expected position of phenprocoumon in the enzyme active site.[111] The 4-hydroxyl group of the coumarin ring was located within hydrogen bonding distance of the two essential catalytic aspartic acid residues (Asp_{25} and $Asp_{25'}$). The two lactone oxygen atoms of the coumarin ring
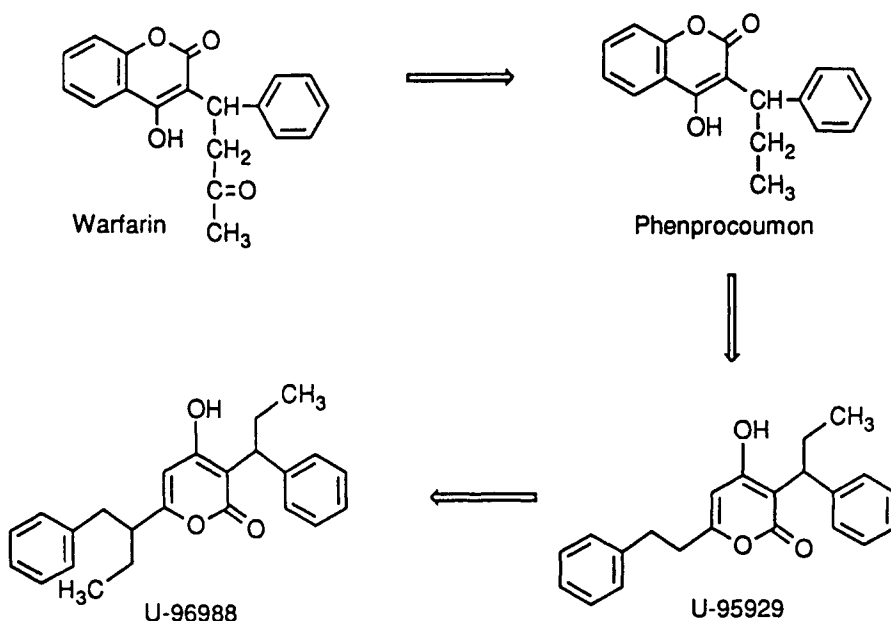
Scheme 3. The development of Upjohn nonpeptide inhibitor U-96988.

were positioned to hydrogen bond with the two NH amides of two isoleucine residues on the flap of the enzyme (Ile_{50} and $Ile_{50'}$). The lactone functional group replaced the water molecule which, as mentioned above, is commonly found in HIV-1 protease/ligand complexes containing various peptidomimetic inhibitors. This ubiquitous water molecule is found in a position to form hydrogen bonds with the two amide carbonyl groups of the ligand, flanking the dipeptide cleavage site, and the NH amides of the two isoleucine residues on the flap of the enzyme. This hydrogen bonding network of 4-hydroxycoumarin defines the essential pharmacophore of this new class of inhibitors. Because of the C_2 symmetry of HIV-1 protease, the phenprocoumon molecule could be found in two orientations related by a 180° rotation. Information from the crystal structure of the phenprocoumon/HIV protease complex has served as the basis for further structure-based design of more potent analogs.

Extension of these studies of structure–activity relationships led to the evaluation of a series of 4-hydroxypyrones, such as U-95929 (K_i = 0.5 µM), as potential inhibitors. Since the α-ethyl group and the α-phenyl ring, at the coumarin C3 position of phenprocoumon, lie approximately in the S_1 and S_2 enzyme pockets, respectively, it was reasoned that the α-position at C6 of the pyrone ring might offer the opportunity to place additional substituents that could be positioned in the S_1' and S_2' pockets. U-96988 (Scheme 3) was then identified as an inhibitor with significant improvement in binding affinity, with K_i values of 38 and 32 nM against HIV-1 and HIV-2 proteases, respectively. The ED_{50} value against HIV-1 infected MT4 or H9 cells was found to be approximately 3 µM. It was also effective against clinical isolates (ED_{50} = 4µM), including AZT resistant isolates. The oral bioavailability of U-96988 in rats and dogs was 80% and 45%, respectively. The half-life after intravenous administration to rats and dogs was 4 and 6 hours, respectively. After extensive preclinical studies, U-96988 entered phase I clinical testing in 1993 as the first in a series of this promising class of nonpeptidic HIV protease inhibitors. Recently a group of investigators at Parke-Davis also described a pyrone-based series of HIV protease inhibitors.[112]

C. Applications of De Novo Design and 3D Database Search for Discovery of HIV-1 Protease Inhibitors: DPM323

The recent report of Lam et al.[113] is an interesting example of de novo drug design and, at the same time, of 3D structure-based drug search of nonpeptidic inhibitors of HIV-1 protease. These investigators focused on the structural water linking the flap residues Ile_{50} and $Ile_{50'}$ to the inhibitor (Figures 2B and 4). The rationale for incorporating the binding feature of the water molecule came from the following considerations made earlier by other research groups[82,100]:

1. Displacement of the water molecule should be energetically favorable; this particular water molecule is unique to retroviral proteases, and incorporation

of a water mimic into an inhibitor should ensure potency and specificity for the HIV-1 protease.

2. Conversion of a flexible, linear inhibitor into a rigid, cyclic structure with restricted conformations should provide a positive entropic effect and thus be an attractive feature of an inhibitor.

3. This principle should also allow the design of low-molecular-weight inhibitors with bioavailability superior to those of peptide-derived inhibitors.

Since diols show high potency in SAR studies,[10,114] a C_2-symmetric diol was incorporated as another feature of inhibitor design. However, Lam et al.[113] had to rely upon computer models of C_2-symmetric diols based on the crystal structure of a hydroxyethylene inhibitor bound to HIV-1 protease.[82] Structures of HIV-1 and HIV-2 proteases complexed to inhibitors containing the diol moiety were published later.[52,65,115] The series of events leading to the design of an inhibitor with properties in accord with what is needed to warrant clinical trials is described in Figure 17, A through K.

Computer models (Figure 17, A and B) were first developed for the C_2 symmetric diols based on the 3D structure of a hydroxyethylene inhibitor bound to HIV-1 protease.[82] Then, intermolecular distances were measured between the symmetric, hydrophobic groups P_1 and P_1', and from P_1 and P_1' to a hydrogen bond donor/acceptor group that binds to the catalytic aspartates (Figure 17C). With this information, a search of the Cambridge Structural Database came up with a "hit" (Figure 17D) that had a phenyl ring containing groups able to interact with aspartates 25 and 25', and an oxygen that mimicked the structural water that originally inspired this inhibitor design strategy. The phenyl ring was first substituted with a cyclohexanone ring to properly position all substituents (Figure 17F), and then the latter was enlarged to a seven-member ring to incorporate the diol moiety (Figure 17G). Finally, the seven-member ring was modified to a cyclic urea because this compound is an excellent hydrogen-bond acceptor and is easy to prepare. Optimization of the stereochemistry was then worked out by modeling studies: the predicted optimal stereochemistry for unnatural D-phenylalanine-derived cyclic ureas with substituents on the nitrogens is 4R,5S,6S,7R (Figure 17 I), and this was confirmed by small-molecule X-ray crystallography. Furthermore, optimization was confirmed by kinetic and biological studies. Modeling studies (Figure 17 J) showed that the N-substitute cyclic urea ring allows (1) proper complementarity between the $P_1/P_1'/P_2/P_2'$ and the corresponding $S_1/S_1'/S_2/S_2'$ pockets; (2) positioning of the cyclic urea oxygen to serve as a mimic for the structural water; and (3) hydrogen binding of the diol moiety to Asp_{25} and $Asp_{25'}$. The DuPont-Merck investigators pointed out that cyclic ureas derived from L- and D-phenylalanine, when unsubstituted at the urea nitrogens, have similarly poor K_i values (= 3000 nM and 4500 nM, respectively). Moreover, if D-phenylalanine was substituted with allyl groups on the nitrogens, the resulting compound, XK-216 shown in Scheme 4, gave a K_i = 4.7 nM, three orders of magnitude more potent than the corresponding L-phenyl-

alanine-derived cyclic urea. The modest antiviral potency showed by XK-216 required other optimization protocols. Modeling studies indicated that the S2/S2' subsites in the enzyme are relatively large and can accommodate aromatic rings; for example, the compound 3 (Scheme 4) containing two β-naphthylmethyl substituents resulted in a 10-fold K_i improvement (K_i = 0.31 nM) over XK-216, but because of its high lipophilic character, the K_i improvement was not paralleled by an increase in antiviral activity. The 3D structure of the HIV-1 protease/compound 3 complex was resolved at 1.8 Å resolution (a stereo drawing of the inhibitor in the active site of HIV-1 protease is shown in Figure 18); it bore out all of the predictions made by modeling: (1) the cyclic urea oxygen replacing the structural water was positioned to make hydrogen bonds with $Ile_{50}/Ile_{50'}$; (2) the diol moiety was within hydrogen bonding distance of both Asp_{25} and $Asp_{25'}$; and (3) a high complementarity between inhibitor and enzyme subsites was evident. Finally, a cyclic urea that has two p-hydroxymethyl benzyl groups as the nitrogen substituents, DMP323 in Scheme 4, showed good bioavailability in animals, a K_i = 0.27 nM toward the enzyme, and what appeared to be excellent antiviral properties in cell culture.
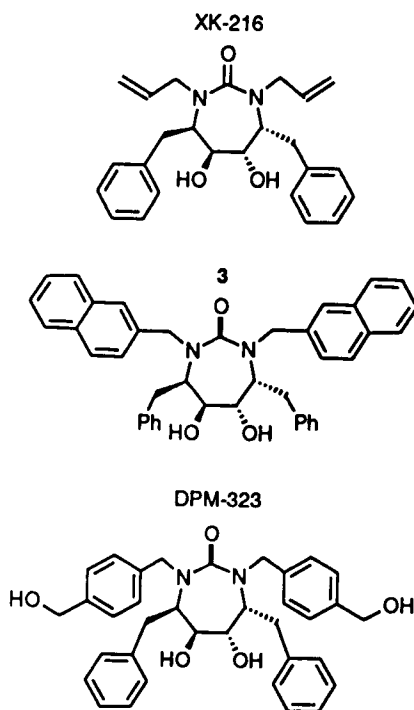
Scheme 4. DuPont-Merck cyclic urea inhibitors XK-216, 3, and DPM-323.
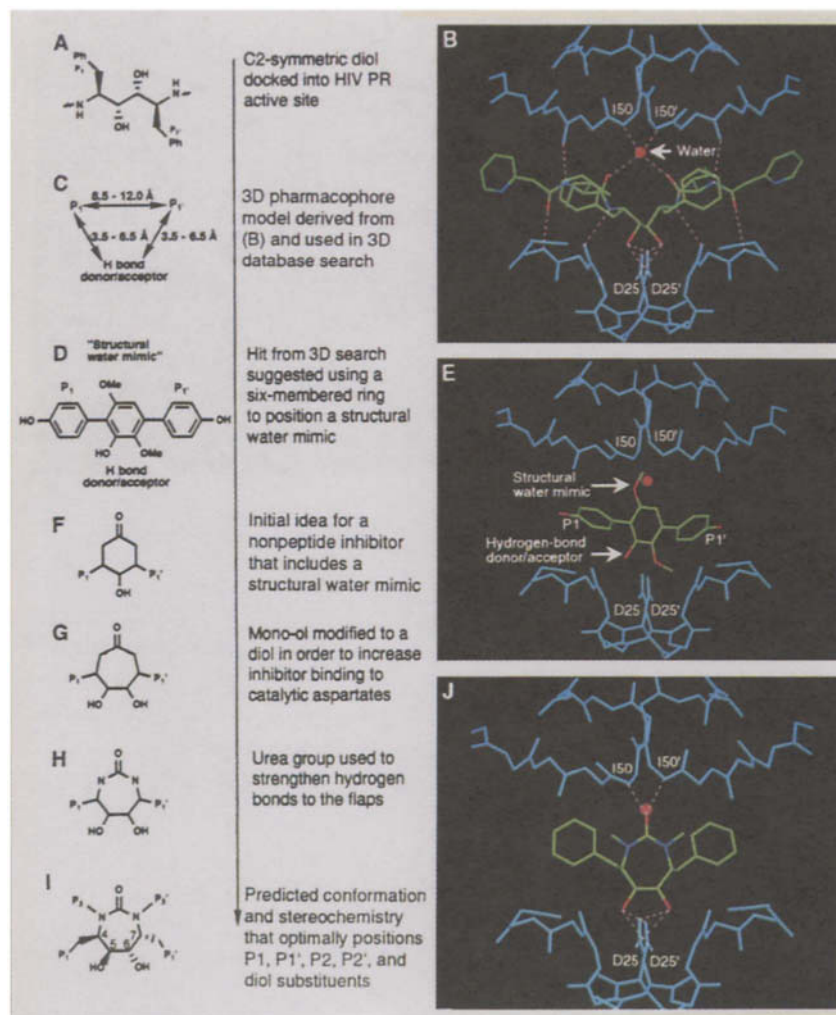
Figure 17. Strategy and steps involved in the design of cyclic urea inhibitors of HIV-1 protease. Reproduced with permission from Lam et al.,[113] *Science* **263**, 380–384. Copyright 1994 by AAAS.

However, as will be discussed later with regard to resistance, the virus was shown to be able to mount considerable resistance against this compound.

In Phase I clinical trials, DMP323 showed poor oral bioavailability in humans and highly variable blood levels, which may be due to its low water solubility and high metabolism of the benzyl moiety. Thus the early preclinical promise of DMP323 was not born out in AIDS patients, and this compound was withdrawn
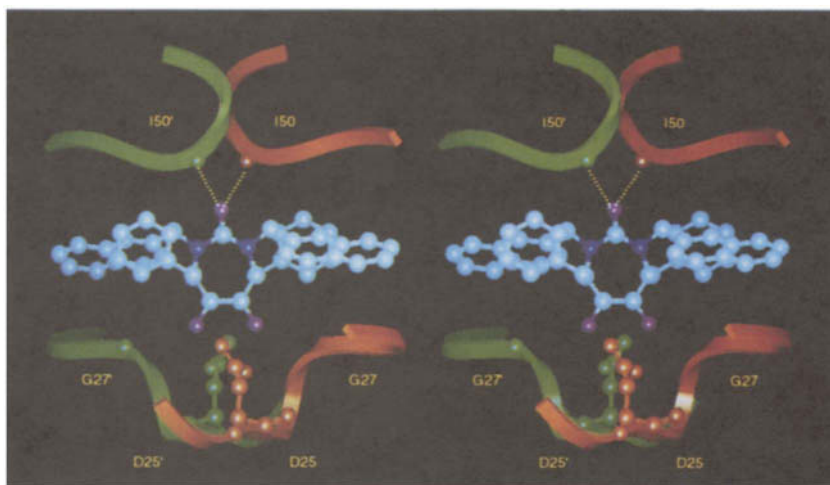
Figure 18. Stereo drawing of the cyclic urea inhibitor 3 in the active site of HIV-1 protease. Reproduced with permission from Lam et al.[113]

from Phase I evaluation. DMP323 is certainly an interesting and perhaps one of the best examples of de novo design and of 3D structure-based drug search, and work on the cyclic isoureas continues at Dupont-Merck. At the same time, the experience with DMP323 also offers an example of how frustrating it can be to apply every rational means at our disposal to make a drug, and then have disappointing results in the clinic.

VII. THE PROBLEM OF DRUG RESISTANCE

The rapid emergence of drug-resistant HIV strains has been a major problem in the development of both nucleoside and non-nucleoside inhibitors of HIV-RT.[5–8] It is only natural to ask whether the same problem might be encountered with HIV-1 protease inhibitors. Indeed, clinical isolates resistant to protease inhibitors have been reported.[116,117] At the same time, a number of studies have begun to unravel selection of drug-resistant HIV-1 protease mutants in cell cultures. In fact, it has been shown in vitro that the virus can select resistant variants to all of the HIV protease inhibitors currently being considered for or already in clinical trials.[118] Here we consider mutants generated in cell cultures treated with some of the inhibitors discussed above. To help with the localization of the amino acids that undergo mutations, we have highlighted them in the 3D structure of HIV-1 protease (Figure 19). Moreover, examples of resistance in clinical trials of select inhibitors will also be discussed.
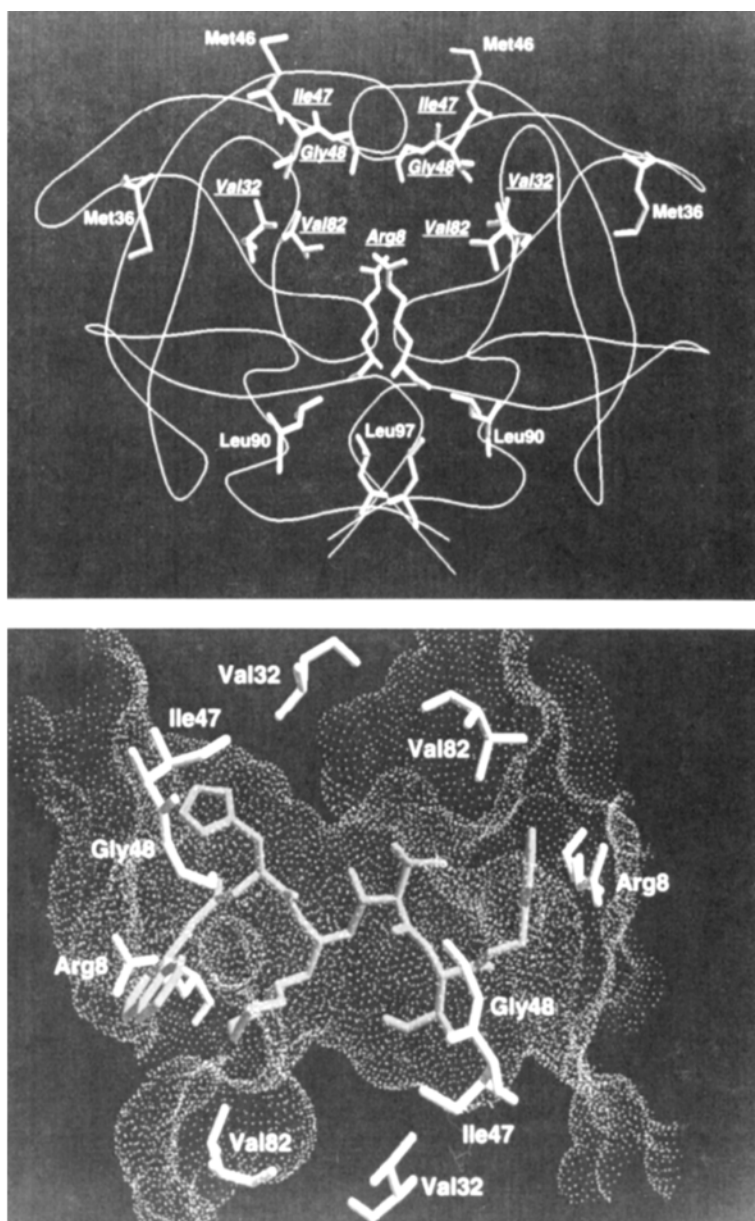
Figure 19. HIV-1 protease amino acids that undergo mutations in cell cultures treated with inhibitors. (**Top**) the amino acids that undergo mutation are represented in the backbone tracing of the enzyme; (**bottom**) those amino acids located at the active site are shown with their van der Waals surfaces interacting with U-75875.

A. A-77003

CEM cells infected with virus were cultured in the presence of increasing concentrations of A-77003.[25] Decreased sensitivity was observed after several passages. A comparison of the sequence of the parent HXB2 clone of HIV-1 with those obtained from day 6 up to 3 months demonstrated that several changes arose during the selection period. The protease mutations that accumulated more frequently were mapped, and the mutated enzymes were cloned and expressed in bacterial systems. The protease mutants were purified from inclusion bodies, reactivated, and tested for their susceptibility to A-77003 (Table 5).

The crystal structure of the HIV-1/A-77003 complex was used to explore the structural basis for reduced drug sensitivity of the various protease mutants. In the wild-type HIV-1 protease, residues $V_{82/82'}$, $V_{32/32'}$, and $R_{8/8'}$ are part of the S_1/S_1', S_2/S_2', and S_3/S_3' subsites, respectively, and their side chains interact with the inhibitor's P_1/P_1', P_2/P_2', and P_3/P_3' groups. The V32I substitution displaces the bulkier Ile in the S_2/S_2' site, allowing little room for movement and causing steric hindrance for the inhibitor. This could account for the observed 3.8-fold increase in the K_i value. Although wild-type and V82I mutant proteases have an apparently similar affinity for the inhibitor, the K_i value for the double mutant V32I/V82I was 21-fold higher than that for the wild-type protease. When the double-mutant enzyme was modeled into the crystal structure of the A-77003/HIV-1 protease complex, a clear increase was seen in steric overlap of the electron clouds of the inhibitor with the new side chains of the enzyme. The synergistic effects of two bulkier substituents did not allow the inhibitor to fit properly at all four subsites, in contrast to what is observed with the wild-type protease.

The R8Q mutation was the most serious among those found for A-77003 because it increased K_i by about 60-fold (Table 5). Modeling of the R8Q mutant into the crystal structure of the A-77003/HIV-1 protease complex indicated decreased van der Waals and charge-induced dipole interactions between the enzyme's two new

Table 5. $K_{i\ app}$ Values for A-77003

	K_{iapp} (nM)
WT	0.53 ± 0.1
V82I	0.54 ± 0.1
M46L	1.34 ± 0.2
M46F	1.95 ± 0.5
V32I	3.80 ± 0.7
V32I/V82I	11.1 ± 1
R8Q	31.1 ± 9

Note: Reproduced with permission from Kaplan et al.[25]

amide groups (substituting two guanidinium groups) and the inhibitor's pyridine groups.

Substitutions with less dramatic effects on K_i values than those discussed above occurred at M46, giving the mutants M46L and M46F, with K_i values for A-77003 three and four times higher, respectively, than that determined for the wild-type protease (Table 5). Ho et al.[119] also studied the effect of A-77003 on HIV-1 drug resistance. However, these investigators used MT-4 cells instead of CEM cells and viral strain NL4-3 instead of HXB2. They found R8Q as the prevalent mutation, but it occurred almost exclusively as a double mutation with M46I. They pointed out that the virus containing only the R8Q mutation grew poorly, whereas the virus containing the R8Q/M46L double mutation in the protease had growth properties equivalent to those of the wild type. Whether the growth characteristics of these mutant viruses reflect changes documented in their proteases is not known. In the 3D structure of the HIV-1 protease, R_8 engages in electrostatic interations with $D_{29'}$ of the other monomer and helps in dimer stabilization. In the R8Q mutant, this interaction is lost and, consequently, enzyme stability is reduced. Kaplan et al.[25]
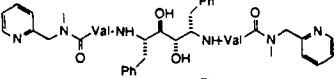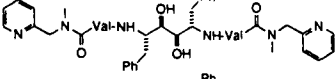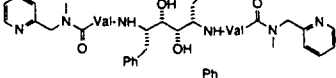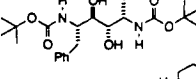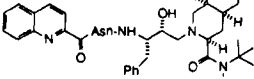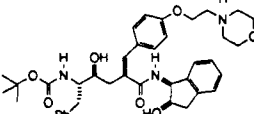
Inhibitor	Structure	Drug Sensitivity ID$_{50}$ (μM)		Drug-Protease Binding K$_I$ (pM)		
		NL4-3	P19	NL4-3	R8Q	M46I
A-77003		0.2	6.0	84	2,700	120
A-76889		0.9	4.7	1,000	23,000	ND
A-76928		0.1	2.8	77	7,200	ND
A-76215		7.0	8.0	22,000	37,000	ND
Ro 31-8959		0.01	0.01	73	270	ND
L-689.502		0.01	0.01	51	140	54

Figure 20. Sensitivity of A-77003 and other inhibitors to parental NL4-3 virus and the same virus after 19 passages (P19) using MT-4 cells, and K_i determinations for the wild-type protease (NL4-3), and mutants R8Q and M46I. No distinction is made in the numbering of residues with regard to their location in one monomer or the other. Reproduced with permission from Ho et al.[119]
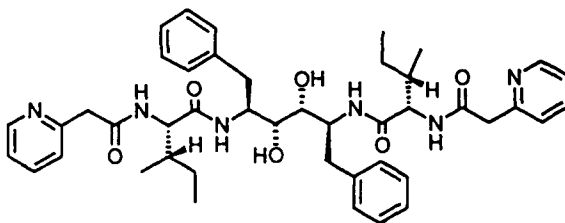
Figure 21. Chemical structure of P9941.

commented that K_m of R8Q protease increased compared to that of the wild type, and Korant[120] reported that the catalytic activity of this mutant was severely depressed. These are two negative kinetic effects that could explain poor growth of the mutant virus. No obvious explanation was found for the restoration of the wild-type growth of the double mutant R8Q/M46I. Ho et al.[119] reasoned that M46, given its position in the flap region, may be involved in binding the viral polyprotein substrates or affects protease activity by modulating the flap dynamics. Crystal-lographic analysis of these mutant proteases may help to explain the relationship, if any, of these mutations to viral growth characteristics. Ho et al.[119] also showed that R8Q protease was resistant to the A-77003 structurally related inhibitors, A-76889 and A-76928 (Figure 20). On the contrary, the C_2 symmetric inhibitor A-76215 (Figure 20), resembling A-77003 but missing both P_3 and P_3' groups, did not show cross-resistance. This was also true for the nonsymmetric inhibitor Saquinavir and L-689,502 (Figure 20), which are not structurally related to A-77003. This broader spectrum of protease mutants susceptable to inhibition by these smaller inhibitors is a good sign; smaller is better!

After serial passage of cells in the presence of the inhibitor P9941 (Figure 21), one that is C_2 symmetric and structurally related to A-77003, Otto et al.[121] were able to select variants of the virus with reduced sensitivity to the inhibitor. Sequences of these viral isolates showed that the protease had the single mutation V82A, which made it about tenfold less sensitive to the inhibitor. Incidently, Ho et al.[119] observed the V82A mutation in their studies with A-77003, but very infre-quently.

B. L-735,524

The Merck investigators[24] made numerous cell culture selections attempts, using both lymphoid cell adapted virus and primary viral isolates from humans, but were unable to isolate any protease mutants that were resistant to L-735,524. However, based upon the 3D structure of HIV-1 protease complexed to an inhibitor closely resembling L-735,524, these workers selected specific substitutions at the active site that, because of their specific location, appeared likely to alter binding of

Table 6. Inhibition by L-735,524 of Mutant HIV-1 Protease

Protease	K_i, nM	k_{cat}/K_m, $sec^{-1} \cdot mM^{-1}$
Wild type (HIV-1)	0.358 ± 0.034	97.07 ± 8.70
Wild type (HIV-2)	3.316 ± 0.300	0.42 ± 0.14
L23I	0.585 ± 0.042	26.67 ± 4.28
L23V	2.812 ± 0.326	4.70 ± 0.28
V32I	2.638 ± 0.249	4.22 ± 1.02
I47V	0.767 ± 0.158	12.04 ± 3.77
I47L	6.615 ± 0.495	0.10 ± 0.01
I50L	0.323 ± 0.070	2.57 ± 0.12
L76M	0.114 ± 0.017	54.95 ± 5.03
V82I	0.052 ± 0.008	138.24 ± 21.53
I84L	0.227 ± 0.018	11.65 ± 0.15
I84V	3.107 ± 0.393	3.16 ± 0.07

Note: Reproduced with permission from Vacca et al.[24]

L-735,524. Consequently, various recombinant proteases carrying those substitutions were made to test their susceptibility to inhibition by L-735,524 (Table 6). Indeed, the effects of the mutation at a specific amino acid residue depended heavily on the substituting group. Certain mutations decreased the enzyme's susceptibility to L-735,524 but also decreased the K_{cat}/K_m; others increased the enzyme susceptibility or had no significant effect (Table 6).

The two mutations V32I and V82I have been documented for several inhibitors. With L-735,524, the V32I mutant shows a ninefold increase in K_i compared to the wild type. Interestingly, the V82I mutant had a K_i sevenfold lower than that of the wild type. Another mutant, L76M, also showed a significant drop in K_i when compared to the wild type (Table 6). It follows that inhibitors like L-735,524 may be especially valuable for therapeutic applications in combination with inhibitors that lead to selection of the V82I and/or the L76M mutations. Studies of structure-guided mutagenesis, like those reported by Vacca et al.,[24] are as important as those on mutants selected by virus passage in the presence of inhibitors. They provide another way of evaluating the weaknesses and strengths of protease inhibitors. Interestingly, Korant[120] reported a bacterial-based screening technique utilizing a library of mutants of the HIV-1 protease that allows rapid identification of drug-resistant protease mutants. He also reported that the most prevalent alterations found with various classes of mutants occurred at V82. The substituting amino acids were variable, and the level of resistance was on the order of tenfold. Other, less frequent mutations at D30 and R8 and in the flap region also produced drug-resistant enzymes. Moreover, mutations at D30 and R8 led to enzymes with highly reduced catalytic activities. This technique allows one to quickly map out the HIV-1 mutated forms resistant to selected inhibitors.

In spite of the fact that the Merck workers were unable to find HIV strains resistant to L-735,524, a recent study by Tisdale et al.[122] documented selection by this compound of resistant viral strains in cell culture and determined the most frequent mutations in the protease to be V32I, M46L, and V82A. Kaplan et al.[25] have stressed variability from selection, and these disparate observations could be due to the application of different selection schemes.

Finally, and most important from the clinical perspective, L-735,524 has shown four- to eightfold reduced sensitivity in a number of patients following 24 weeks or more of therapy.[116] This reduction was accompanied by various protease-associated amino acid substitutions; among these, mutations at Val_{82} were present in all resistant strains (V82A/T/F). The mutation V82I, shown to actually decrease K_i (Table 6), was not seen. Another protease mutation documented in infected patients was I84V, one that has been shown (Table 6) to increase K_i for the protease by a factor of 10.

C. DMP323

The DuPont-Merck investigators found a fourfold increase in the apparent IC_{90} of DMP323 against the virus obtained after serial passage in cell culture in the presence of escalating concentrations of the linear diol inhibitor P9941. The mutation in the protease gene was V82A.[121] In additional studies, passage of virus in cell culture in the presence of DMP323 resulted in the selection of mutants with V82F/L97V (tenfold resistant) or V82F/I84V (100-fold resistant).[123,124]

D. Saquinavir

A number of studies have begun to characterize in vitro selection of HIV-1 mutants that are resistant to this inhibitor. In C8166 cells infected with HIV-IIIB, resistant viruses were obtained after five passages in the presence of Saquinavir.[125] The most resistant strain showed an ED_{50} value of 21.8 nM, which is a log unit higher than that against the wild-type strain. In CEM cells infected with HIV-1 strain GB8, resistant viruses were observed after seven to nine passages in the presence of increasing concentrations of Saquinavir. In parallel experiments with two inhibitors of reverse transcriptase, AZT and TIBO, resistance to the protease inhibitor Saquinavir arose less readily and required more passages.[126]

As a result of the studies described above, Jacobsen et al.[127] investigated the molecular basis of the resistant phenotype for the Roche compound. After 11 passages of the HIV-1 GB8 in CEM cells in the presence of Saquinavir, they observed a substantial decrease in the sensitivity of the virus to this inhibitor. The most prevalent change was the double mutation G48V/L90M. The virus containing this mutation in the protease showed a 40-fold increase in IC_{50}. G_{48} is part of the I_{47} $GGIGG_{52}$ flap motif that embraces the inhibitor. The 3D model of the HIV-1 protese showed that the side chain of Val_{48} reaches the inside of the active site and creates steric hindrance problems for the inhibitor. Leu_{90} is in the core of the enzyme

and does not interact with the inhibitor. However, in modeling studies based on the crystal structure of the wild-type HIV-1 protease complexed to the inhibitor Ro XI, Maschera et al.[128] found that the L90M mutation affects the structure of the active site. This is because the thioether methyl group is approximately 0.6 Å closer to the main chains of Asps 25 and 25′ than either of the methyl groups on the branched side chain of Leu_{90}.

Jacobsen et al.[117] also investigated the question of resistance to Saquinavir in the clinic. After about 1 year of treatment with 600 mg of Saquinavir (t.i.d.) alone, or in combination with AZT, the most frequent mutations observed were, again, G48V and L90M, although the combined double mutation G48V/L90M was rare. Less frequent mutations, e.g., at positions 36 (M36I) and 71 (A71V/T), previously observed in vitro were also documented in vivo.

E. SC-52151

Potts et al.[129] reported resistant HIV-1 variants after several passages of clinical and laboratory HIV-1 strains in T cell lines or in peripheral blood mononuclear cells in the presence of increasing concentrations of SC-52151 or its structurally related analog, SC-55389A. The IC_{50} values for the resistant HIV-1 variants were more than tenfold higher than the untreated controls. The principal mutation for SC-52151 was G48V, and for SC-55389A, V82A. Sequential exposure of the virus to the two drugs gave the N88S mutation in the protease and virus cross-resistant to both compounds. Whereas we have already discussed the structural implications of mutations at positions 48 and 82, the change at 88 is new. Potts et al.[129] suggested that Asn_{88} is part of a structurally conserved helical domain present in both monomeric and dimeric aspartyl proteases. Mutations at Asn_{88} may disrupt important hydrogen bond interactions among Asn_{88}, Thr_{31}, Thr_{74}, and Asp_{29}, and thus influence the S_2/S_2' inhibitor-binding subsites.

F. Inhibitor Cross-Resistance

Tisdale et al.[122] used a panel of six HIV protease inhibitors, including L-735,524, Saquinavir, A-77003, DPM323, AG-1284, and VB 11-328, to test their ability to select resistant virus in cell culture. HIV-1 (clone HXB2) was grown in MT4 cells in the presence of escalating concentrations of each inhibitor. After six passages, a decreased inhibitor sensitivity ranging from 3- to 40-fold was observed. DNA sequence analysis revealed one to four mutations in the protease gene; these are shown in Table 7. When the six passaged virus variants were tested against the six protease inhibitors there was a great deal of cross-resistance, but interestingly, not all were cross-resistant. For example, the mutation I84V generated by DPM323 was cross-resistant with all other inhibitors, whereas mutation V32I/V82A generated by L-735,524 gave cross-resistance with all of the other inhibitors except for Saquinavir. G48V/L90M generated by Saquinavir was cross-resistant with L-735,524 and A-77003, but not with the other three inhibitors. Therefore, therapy

Table 7. HIV-1 Protease Mutations Individually Selected
by Six HIV-1 Protease Inhibitors in Cell Culture[122]

Compound	HIV-Protease Mutation
Saquinavir	G48V, L90M
A-77003	R8Q/K, M46I, G48V
DPM323	L10F, K45I, I84V
L-735,524	V32I, M46L, V82A
Ag-1284	A71V/T, V77I, V82A, T91A
VB11,328	I50V, M46I, I47V

with a single protease inhibitor would appear to have little chance of success and combination drug therapy would be the most obvious choice.

G. Summary on Resistance

One of the reasons our laboratory embarked on studies of the HIV-1 protease as a target in AIDS therapy was our feeling that resistance, which has plagued RT inhibitors in the clinic, would not present a problem for the protease. This hope was based, in part, upon the small size of the enzyme. With such a parsimonious structure, it seemed unlikely that many changes in sequence could be tolerated and still preserve the catalytic activity. Moreover, the enzyme must hydrolyze eight distinct peptide bonds in the polyprotein precursor, and it has been argued that this processing is temporally regulated by the sequences in the substrate. An alteration in protease sequence that preserved catalytic activity, but which modified specificity, therefore, could be equally devastating to viral maturation and formation of infective virus. It came as a disappointment to workers in the protease field that, in fact, protease inhibitors, as well as RT inhibitors, can select for resistant variants of the virus, so our original supposition was incorrect. Furthermore, although in general the degree to which resistance occurs with protease inhibitors is orders of magnitude less than encountered with RT inhibitors, cases are known in which resistance is associated with increases in K_i of 1000-fold.

What are some of the problems in interpreting results on resistance? First of all, most of the results come from studies in cell culture, where the concentration of inhibitor is maintained at a much higher level throughout the series of cell passages than can be achieved in AIDS patients. Nevertheless, we often see that a given inhibitor generates the same mutations in cell culture and in patients.[116,117] Then there is the question of reaching a protease target in a newly emerged virion where the concentration of the enzyme is enormously high, perhaps millimolar. Can we achieve levels of the protease inhibitor needed, and what is the stoichiometry in these particles? Furthermore, although we are looking carefully at mutations in the protease itself, changes in other genes could alter the growth and stability of the

virus and these could be interpreted as protease-resistant mutants. Of course, cells have the ability to develop drug resistance on their own through the aegis of the multidrug resistance channel. This could also have an impact on observations in vitro and in vivo.

With this in mind, what have we learned thus far from these studies on resistance?

1. HIV-1 variants with reduced susceptibility clearly are selected as a consequence of passage in cell cultures treated with HIV-1 protease inhibitors, and resistance has now been documented in the clinic. All of these inhibitor-resistant viral strains carried a protease with one or more mutations that could be easily identified. These changes are seen in various parts of the enzyme structure, but those in the catalytic region are particularly frequent. The mutated proteases showed increased K_i, generally of 10- to 100-fold compared to the wild-type enzyme. Clearly there exist "hot spots"[118] in the protease that can be altered in such a way as to sustain the needed function of the enzyme in processing the viral polyproteins while in the presence of the particular drug that selected for the mutation in question. Key "hot spots" include R_8, V_{32}, G_{48}, V_{82}, V_{84}, and L_{90}.

2. Different HIV-1 protease inhibitors may or may not select different mutations. Moreover, the same inhibitor may select different sequence variants. Kaplan et al.[25] suggested the need to use several selection schemes to observe the range of variation that will affect sensitivity.

3. The potency of certain inhibitors may be preserved, decreased, or even increased in going from wild-type enzyme to mutants selected by structurally different inhibitors. For example, Saquinavir does not show cross-resistance to the R8Q protease mutant selected by A-77003, whereas the mutation G48V/L90M generated by Saquinavir also decreased the inhibition power of L-735,524 and A-77003. On the other hand, L735,524 potency against the V82I mutant (selected by various inhibitors) is increased sevenfold. Taken together, these observations certainly support combination therapy.

4. Finally, it will be important to identify and kinetically characterize the protease mutants selected by various inhibitors. New "drug-resistant" strains selected in culture often have proteases that are catalytically less efficient than wild-type enzyme, yet are able to sustain infectious virions. Information such as this must be correlated with the growth characteristics of the mutant viruses to generate an important database for the design and testing of new inhibitors and/or combinations of them that can take advantage of the mutations.

VIII. CONCLUSIONS AND PERSPECTIVES

We have described a series of inhibitors of the HIV-1 protease, each of which meets one or more basic criteria: (1) potency against the enzyme, (2) potency against the virus in cell culture, and (3) reasonable pharmacokinetics in vivo. Because of these properties, most of the inhibitors emphasized in this review have entered clinical

trials; we all anxiously await the results of their performance in AIDS patients. Meanwhile, chemical elaboration of these compounds to improve upon their properties and the search for new chemical entities continue, powered by the myriad interrelated approaches and ingenuity emphasized in this narrative.

Besides the more intensively explored protease and reverse transcriptase, other viral targets are on the horizon or under consideration. A compound, Ro 24-7429, directed to block the action of *tat*, an activator of viral transcription, entered clinical trials in 1992. Antisense oligonucleotides were used successfully in cell cultures to block HIV-1 replication,[130] and studies with ribozymes, catalytic RNAs, to cleave specific HIV sequences are progressing.[131] Gene therapy is another approach being pursued, and a clinical trial directed at blocking the action of the Rev protein, a regulatory factor, has been proposed. In addition to the development of new molecules to counteract the action of the viral protease and RT and the aforementioned evaluation of new targets and new therapies, major emphasis is being placed on the use of combinations of RT and protease inhibitors in AIDS therapy. Moreover, as it is now well established that viral particles are continually produced at all stages of progression to AIDS,[132–134] an essential component of clinical trials is to determine at which point an individual therapy is most effective.

Yet, targeting the virus alone may not be enough. Even in the best-case scenario, where we succeed in optimizing treatment protocols with antiviral agents, the best we can expect is to be able to curb the viral load at any time during the course of the disease and delay the onset of opportunistic infections. A cure for AIDS implies the elimination of every single infected cell from the body and restoration of immune function; this is still beyond the reach of present science. Prevention, of course, would even be more desirable than a cure, but the intense, worldwide effort in this area to produce a vaccine or to alter behavior has been largely disappointing.[135,136] So, while the prodigious attempt to develop an effective antiviral is in progress, we have to turn to basic science[137] and answer the fundamental question: How does HIV cause AIDS? The need for a detailed elucidation of the critical steps involved in the virus–host relation from infection to the onset of the AIDS symptoms is a major prerequisite for developing therapies more effective than, and synergistic to, those described here.

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NOTE ADDED IN PROOF

We wish to emphasize that since submission of this chapter, there have been expected, significant developments in the clinical testing of HIV-1 protease inhibitors. Most important, in November, 1995 the Roche compound Ro-31 8959 (Saquinavir) was recommended for approval by the FDA. Approval was given not as a monotherapy, but in combination with other nucleosides. Another compound cited here, Merck L-735524 (Crixivan), is in Phase III clinical trial, as well as the Abbott compound ABT-538 (Ritonavir). We also would like to point out that, though the name Upjohn is used throughout the text, as of November 1995 we have become a new company, Pharmacia & Upjohn.

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