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NEW ANTI-HIV AGENTS IN PRECLINICAL OR CLINICAL DEVELOPMENT

Erik De Clercq

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ABSTRACT

Virtually all the compounds that are currently used (or have been the subject of advanced clinical trials) for the treatment of HIV infections, belong to one of the following classes: (i) nucleoside reverse transcriptase inhibitors (NRTIs): i.e., zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, emtricitabine and nucleotide reverse transcriptase inhibitors (NtRTIs) (i.e., tenofovir disoproxil fumarate); (ii) non-nucleoside reverse transcriptase inhibitors (NNRTIs): i.e., nevirapine, delavirdine, efavirenz, emivirine; and (iii) protease inhibitors (PIs): i.e., saquinavir, ritonavir, indinavir, nelfinavir, amprenavir and lopinavir. In addition to the reverse transcriptase and protease reaction, various other events in the HIV replicative cycle can be considered as potential targets for chemotherapeutic intervention: (i) viral adsorption, through binding to the viral envelope glycoprotein gp120 (polysulfates, polysulfonates, polycarboxylates, polyoxometalates, polynucleotides, and negatively charged albumins); (ii) viral entry, through blockade of the viral coreceptors CXCR4 (i.e., bicyclam (AMD3100) derivatives) and CCR5 (i.e., TAK-779

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derivatives); (**iii**) virus-cell fusion, through binding to the viral envelope glycoprotein gp41 (T-20, T-1249); (**iv**) viral assembly and disassembly, through NCp7 zinc finger-targeted agents (2,2'-dithiobisbenzamides (DIBAs), azadicarbonamide (ADA)); (**v**) proviral DNA integration, through integrase inhibitors such as 4-aryl-2,4-dioxobutanoic acid derivatives; (**vi**) viral mRNA transcription, through inhibitors of the transcription (transactivation) process (flavopiridol, fluoroquinolones). Also, various new NRTIs, NNRTIs and PIs have been developed that possess, respectively: (**i**) improved metabolic characteristics (i.e., phosphoramidate and cyclosaligenyl pronucleotides by-passing the first phosphorylation step of the NRTIs), (**ii**) increased activity ("second" generation NNRTIs (i.e., TMC-125, DPC-083)) against those HIV strains that are resistant to the "first" generation NNRTIs, or (**iii**), as in the case of PIs, a different, modified peptidic (i.e., azapeptidic (atazanavir)) or non-peptidic scaffold (i.e., cyclic urea (mozenavir), 4-hydroxy-2-pyrone (tipranavir)). Non-peptidic PIs may be expected to inhibit HIV mutant strains that have become resistant to peptidomimetic PIs.

ABBREVIATIONS

HIV: human immunodeficiency virus;
NRTIs: nucleoside reverse transcriptase inhibitors;
NtRTIs: nucleotide reverse transcriptase inhibitors;
NNRTIs: non-nucleoside reverse transcriptase inhibitors;
PIs: protease inhibitors;
DIBA: 2,2'-dithiobisbenzamide;
ADA: azadicarbonamide;
AIDS: acquired immune deficiency syndrome;
HSV: herpes simplex virus;
STD: sexually transmitted disease;
MIP-1 α and -1 β : macrophage inflammatory proteins;
SDF-1: stromal-cell derived factor;
PBMCs: peripheral blood mononuclear cells;
TM4: transmembrane segment;
SI: syncytium-inducing; NSI, non-syncytium-inducing;
NOBA: 3-nitrosobenzamide;
AZT: zidovudine;
ddI: didanosine;
ddC: zalcitabine;
d4T: stavudine;
3TC: lamivudine;

ABC: abacavir;

bis(POM)-PMEA: bis(pivaloyloxymethyl)-9-(2-phosphonylmethoxyethyl)adenine, adefovir dipivoxyl;

bis(POC)-PMPA: bis(isopropylloxycarbonyloxymethyl)-(*R*)-9-(2-phosphonylmethoxypropyl)adenine, tenofovir disoproxil;

dOTC: (\pm)2'-deoxy-3'-oxa-4-thiocytidine;

(-)-FTC: emtricitabine;

DAPD: amdoxovir, (-)- β -D-2,6-diaminopurine dioxolane;

bis(SATE)ddAMP: bis(S-acetyl-2-thioethyl)phosphotriester of ddA.

I. INTRODUCTION

Combination therapy, comprising at least three anti-HIV drugs, has become the standard treatment of AIDS or HIV-infected patients. Virtually all drugs that have been licensed for clinical use for the treatment of HIV infections fall into one of the following three categories:

- (i) nucleoside and nucleotide reverse transcriptase inhibitors (termed NRTIs and NtRTIs, respectively), that, following three phosphorylation steps (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir) or two phosphorylation steps (tenofovir) act, as chain terminators and/or inhibitors, at the substrate binding site of the reverse transcriptase;
- (ii) non-nucleoside reverse transcriptase inhibitors (NNRTIs), that interact with the reverse transcriptase at an allosteric, non-substrate binding site (nevirapine, delavirdine, efavirenz); and
- (iii) protease inhibitors (PIs), that specifically inhibit, as peptidomimetics, the virus-associated protease (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir). Guidelines to the major clinical trials with these compounds have been recently published.¹

Although the long-term goal of eradicating the virus from latently and chronically infected cells remains forbidding,² the advent of so many new compounds, other than those that have been formally approved, for the treatment of HIV infections, will undoubtedly improve the prognosis of patients with AIDS and AIDS-associated diseases. Here I will primarily address those new anti-HIV compounds that have emerged as promising anti-HIV drug candidates during the past few years and are in preclinical or early clinical development, and that are targeted at well-defined steps in the HIV replicative cycle. The HIV life cycle can be schematically divided into ten steps (Fig. 1) and most of

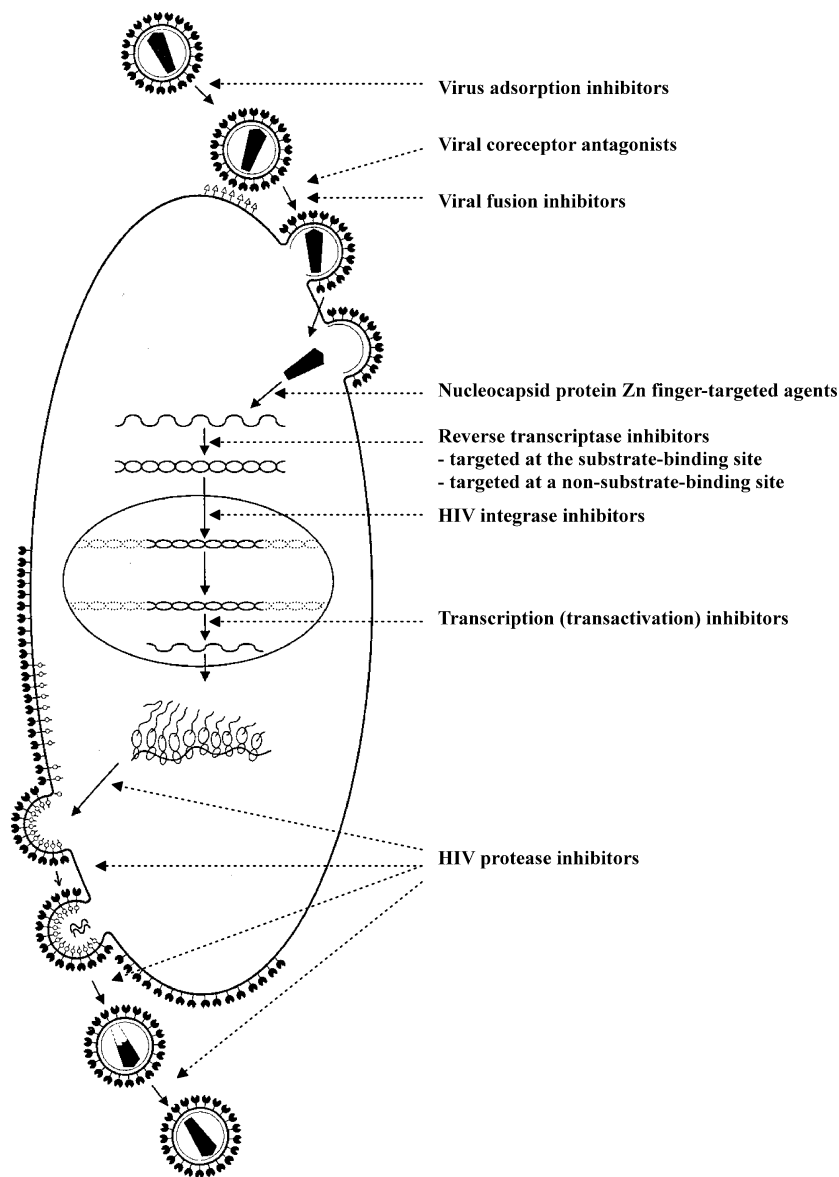


Figure 1. HIV life cycle depicting the molecular targets with which anti-HIV agents interact.

these steps have been envisaged as targets for chemotherapeutic intervention. The compounds to be discussed have been grouped in different categories (Fig. 1) according to the target(s) with which they interact.

As the emergence of resistance towards currently used anti-HIV drugs (NRTIs, NNRTIs and PIs) is an important determinant in the eventual drug failure, new drug development strategies are attempting at circumventing the virus-drug resistance problem, by focussing on either novel targets (other than the reverse transcriptase and protease) or new compounds capable of suppressing HIV strains that are resistant to the currently used reverse transcriptase or protease inhibitors. Mutations (located in the reverse transcriptase or protease) that are associated with resistance (or reduced susceptibility) to the NRTIs, NtRTIs, NNRTIs and PIs, and to the new virus entry inhibitor T-20 (enfuvirtide) are listed in Table 1.³

Table 1.

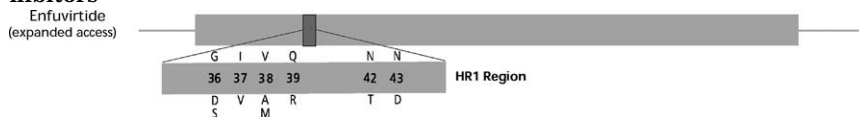
Mutations in the Reverse Transcriptase Gene Associated with Reduced Susceptibility to Reverse Transcriptase Inhibitors												
Nucleoside Reverse Transcriptase Inhibitors												
Multi-nRTI Resistance: 151 Complex	A		V F		F		Q					
	62		75 77		116		151					
Multi-nRTI Resistance: 69 Insertion Complex	M		V		Y		M					
	41		I L									
Multi-nRTI Resistance: NAMs	M		D		V		L T K					
	41		67 69 70		118		210 215 219					
Zidovudine	M		D		V		L T K					
	41		67 70		118		210 215 219					
Stavudine	M		D		V		L T K					
	41		67 70		118		210 215 219					
Didanosine	K		L									
	65		74									
Zalcitabine	K		T				M					
	65		69 74				184					
Abacavir	K		L		Y		M					
	65		74		115		184					
Lamivudine	E				V		M					
	44				118		184					

(continued on next page)

Table 1. — continued from previous page

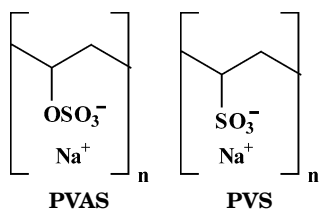
Nucleotide Reverse Transcriptase Inhibitor														
Tenofovir														
			K											
			65											
			R											
Nonnucleoside Reverse Transcriptase Inhibitors														
Multi-NNRTI Resistance														
Multi-NNRTI Resistance: Accumulation of Mutations														
Nevirapine														
Delavirdine														
Efavirenz														
Mutations in the Protease Gene Associated with Reduced Susceptibility to Protease Inhibitors														
Protease Inhibitors														
Multi-PI Resistance: Accumulation of Mutations														
Indinavir														
Ritonavir														
Saquinavir														
Nelfinavir														
Amprenavir														
Lopinavir/ Ritonavir														
Atazanavir (expanded access)														

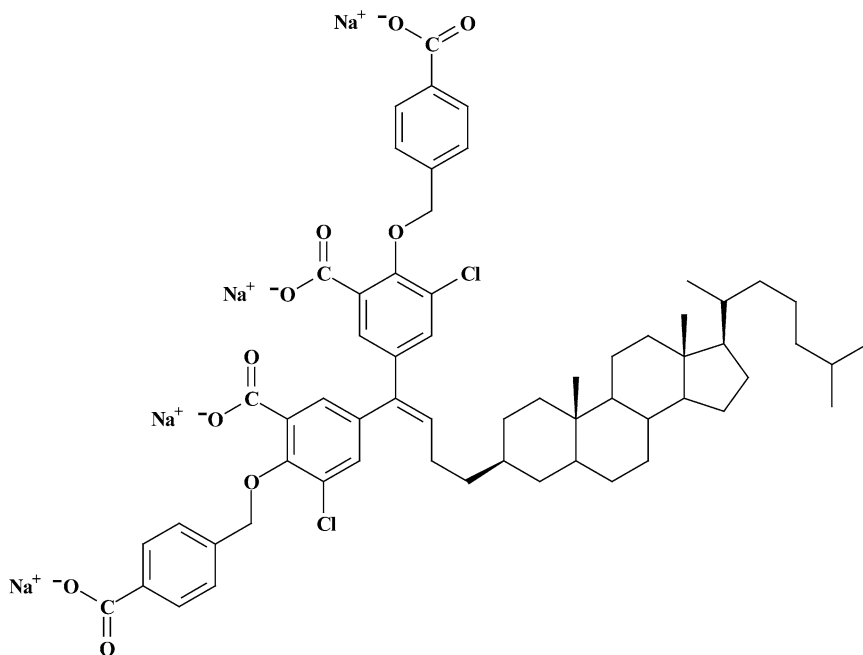
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Table 1. — continued from previous page**Mutations in the gp41 Envelope Gene Associated with Resistance to Entry Inhibitors****II. VIRUS ADSORPTION (gp120) INHIBITORS**

A great variety of polyanionic compounds have been described to block HIV replication through interference with virus adsorption (or binding) to the cell surface: i.e., polysulfates (for example, polyvinylalcohol sulfate (PVAS) (**1**)), polysulfonates (for example, polyvinyl sulfonate (PVS) (**1**)), polycarboxylates, polyphosphates, polyphosphonates and polyoxometalates. Noteworthy among the polyanionic substances are the cosalane analogues (**2**) containing the polycarboxylate pharmacophore,⁴ as well as the sulfated polysaccharides extracted from sea algae.⁵

All these compounds, whether synthetic or of natural origin, are assumed to exert their anti-HIV activity by shielding off the positively charged sites in the V3 loop of the viral envelope glycoprotein (gp120)^{6,7} which is necessary for virus attachment to the cell surface heparan sulfate, a primary binding site, before a more specific binding occurs to the CD4 receptor of the CD4⁺ cells, and to the CXCR4 coreceptor of the CXCR4⁺ cells (the latter in the case of X4 and dual tropic X4/R5 HIV strains). Heparan sulfate is widely expressed on animal cells and, as it is involved in the virus-cell binding of a broad spectrum of enveloped viruses, including herpes simplex virus (HSV),⁸ dengue virus⁹ and other flaviviruses (i.e., Japanese encephalitis virus),¹⁰ it

**Scheme 1.**

**Cosalane analogue*****Scheme 2.***

also explains why polysulfates have a broad-spectrum antiviral activity against HIV, HSV and various other enveloped viruses.¹¹

The major role of polysulfates or polyanionic substances in general in the management of HIV infections may reside in the prevention of sexual transmission of HIV infection, as these compounds, if applied as a vaginal formulation, may successfully block HIV infection through both virus-to-cell and cell-to-cell contact. These compounds therefore merit being pursued as vaginal microbicides.

Also qualifying as a potential microbicide is cyanovirin-N (**3**), a 11-kDa protein originally isolated from the cyanobacterium *Nostoc ellipsosporum*;¹² cyanovirin-N blocks both CD4-dependent and CD4-independent binding of soluble gp120 to target cells, and dissociates bound gp120 from target cells.¹³ This supports the potential utility of cyanovirin-N as a candidate microbicide to prevent the sexual transmission of HIV.

The fact that in addition to their anti-HIV activity, polyanionic substances such as poly[sodium(4-styrene)sulfonate], also inhibit other

(H₂N)Leu—Gly—Lys—Phe—Ser—Gln—Thr—Cys—Tyr—Asn—
 —Ser—Ala—Ile—Gln—Gly—Ser—Val—Leu—Thr—Ser—Thr—Cys—Glu—Arg—
 —Thr—Asn—Gly—Gly—Tyr—Asn—Thr—Ser—Ser—Ile—Asp—Leu—Asn—
 —Ser—Val—Ile—Glu—Asn—Val—Asp—Gly—Ser—Leu—Lys—Trp—Gln—Pro—
 —Ser—Asn—Phe—Ile—Glu—Thr—Cys—Arg—Asn—Thr—Gln—Leu—Ala—
 —Gly—Ser—Ser—Glu—Leu—Ala—Ala—Glu—Cys—Lys—Thr—Arg—Ala—
 —Gln—Gln—Phe—Val—Ser—Thr—Lys—Ile—Asn—Leu—Asp—Asp—His—Ile—
 —Ala—Asn—Ile—Asp—Gly—Thr—Leu—Lys—Tyr—Glu(COOH)

Cyanovirin-N

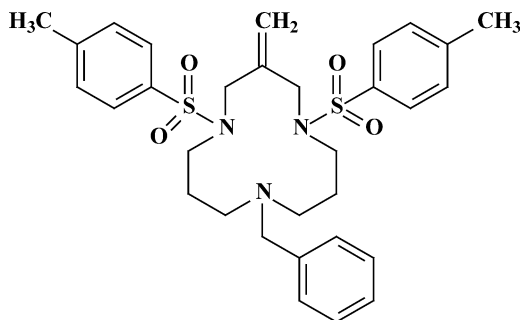
Scheme 3.

sexually transmitted disease (STD) pathogens, i.e., herpes simplex virus, *Neisseria gonorrhoeae* and *Chlamydia trachomatis*,¹⁴ further adds to their potential therapeutic and preventive value.

The attachment of the HIV surface glycoprotein gp120 to CD4⁺ cells, the first step in virus entry, can also be blocked by CD4-immunoglobulin G2 (CD4-IgG2, PRO 542), a recombinant antibody-like fusion protein wherein the heavy- and light-chain variable domains of human IgG2 have been replaced with the D1D2 domains of human CD4.¹⁵ Unlike monovalent and divalent CD4-based proteins, tetravalent PRO 542 potentially neutralizes diverse primary HIV-1 isolates: it has the potential for cross-linking HIV-1 gp120-gp41 trimers on the virion surface.¹⁶ Synergistic activity in blocking virus-cell and cell-cell fusion was achieved upon combination of PRO 542 with the fusion inhibitor T-20 (see *supra*).¹⁷ Preliminary evidence of antiviral activity, as monitored by a reduction in plasma viral load, was noted after (single) intravenous infusion of PRO 542 in HIV-infected individuals.¹⁸

Viral entry can also be blocked by linear synthetic peptides corresponding to the central 15–21 amino acid sequence of the gp120 V3 loop, such as the R15K peptide.¹⁹ These V3-derived peptides inhibit the interaction of gp120 with the host cell surface glycosphingolipids. Importantly, daily infusions of R15K for 2 weeks (10 mg/(kg day)) in rhesus macaques inoculated with the simian human hybrid virus SHIV significantly reduced viral load and prevented chronic infection.¹⁹

Recently, a new chemical entity, namely cyclotriazadisulfonamide (CADA) (**4**), was identified that inhibited HIV infection through down-modulating CD4 receptor expression.²⁰ The antiviral activity of a large set of varying CADA derivatives correlated closely with their ability to down-modulate the CD4 receptor.²¹ CADA did not alter the expression of any other cellular receptor (i.e., CXCR4, CCR5, etc.) examined so far. Time course experiments established that CADA in its



CADA

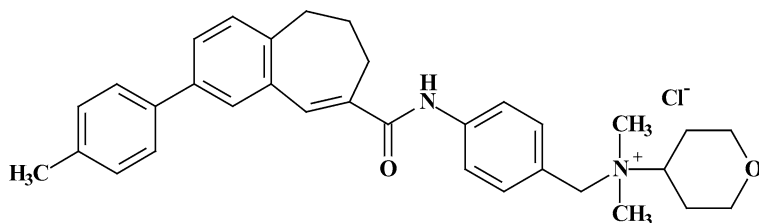
Scheme 4.

mechanism of action differs from any other CD4-interacting agent described previously. It is assumed to down-regulate CD4 expression at the (post)translational level.²¹

III. VIRAL CORECEPTOR ANTAGONISTS

To enter cells, following binding with the CD4 receptor, the HIV-1 particles must interact, again through the viral envelope glycoprotein gp120, with the CXCR4 coreceptor²² or CCR5 coreceptor.²³ CXCR4 is the coreceptor for HIV-1 strains that infect T-cells (T-tropic or X4 strains), and CCR5 is the coreceptor for HIV-1 strains that infect macrophages (M-tropic or R5 strains). CXCR4 and CCR5 have not evolved simply to act as coreceptors for HIV entry; they normally act as receptors for chemokines (chemoattractant cytokines). The normal ligands for CCR5 are RANTES (“regulated upon activation, normal T-cell expressed and secreted”) and MIP-1 α and -1 β (“macrophage inflammatory proteins”), whereas for CXCR4 only one natural ligand, namely SDF-1 (“stromal-cell derived factor”) has been identified.

Of these chemokines, the LD78 β isoform of MIP-1 α has emerged as the most potent chemokine for inhibiting HIV-1 infection in peripheral blood mononuclear cells (PBMCs)^{24,25} as well as monocytes/macrophages.²⁶ LD78 β variants, i.e., with appropriate amino acid substitution(s) such as D6A and P8A, have been observed that retain their chemokine functions, while acquiring more potent ability to suppress R5 HIV-1 replication.²⁷



TAK-779

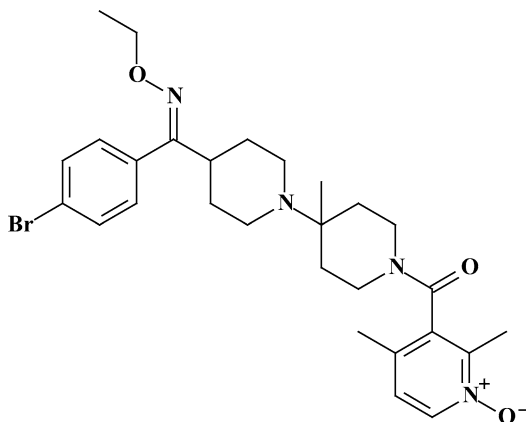
Scheme 5.

TAK-779, a quaternary ammonium derivative (**5**) is the first non-peptidic molecule that has been described to block the replication of M-tropic R5 HIV-1 strains at the CCR5 level.²⁸

A binding site for TAK-779 has been identified within the transmembrane helices 1, 2, 3 and 7 of CCR5.²⁹ TAK-779 has been found to inhibit R5 HIV-1 strains in the nanomolar concentration range, while not affecting X4 HIV-1 strains at 10,000-fold higher concentrations.²⁸ TAK-779 is not a “pure” CCR5 antagonist, as it also demonstrates some antagonism towards CCR2b. Unlike RANTES, TAK-779 does not induce internalization of CCR5. The clinical potential of TAK-779 and its congeners³⁰ in the therapy and/or prophylaxis of HIV-1 infections, remains to be further explored.

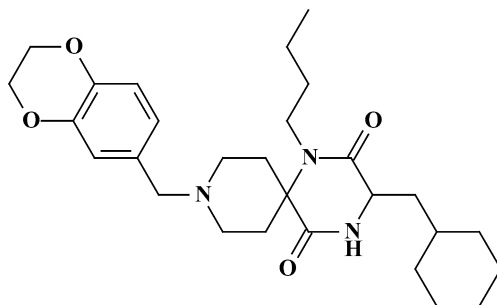
Following TAK-779, a number of piperidinylpiperidine derivatives, i.e., 4-[(*Z*)-(4-bromophenyl)-(ethoxyimino)methyl]-1'-[(2,4-dimethyl-3-pyridinyl)-carbonyl]-4'-methyl-1,4'-bipiperidine N-oxide (SCH 351125),³¹ and piperazinylbipiperidine derivatives, i.e. 1-[(2,4-dimethyl-3-pyridinyl)carbonyl]-4-methyl-4-[3(*S*)-methyl-4-[1(*S*)-[4-(trifluoromethyl)phenyl]-ethyl]-1-piperazinyl]-piperidine N1-oxide (SCH 350634),³² have been described as potent and selective CCR5 antagonists. They block the binding of RANTES to CCR5, as well as the replication of R5 HIV-1 strains, within the nanomolar concentration range.

SCH 351125 (SCH-C) (**6**) also strongly inhibits the replication of R5 HIV-1 in SCID-hu Thy/Liv mice, shows a favorable pharmacokinetic profile (i.e., good oral bioavailability in rats, dogs and monkeys), and has been selected as the drug candidate for further human clinical trials.³³ Preliminary clinical data, based on the oral administration of 25 mg SCH-C twice daily for 10 days to 12 adults infected with HIV-1 and currently on no antiretroviral agents, indicated that SCH-C is able to achieve a 0.5–1.0 log₁₀ reduction in viral load.³⁴



SCH-C
SCH 351125

Scheme 6.

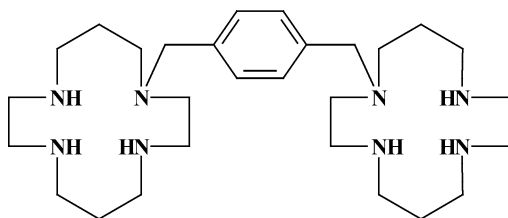


E913

Scheme 7.

SCH-C has been found to act synergistically with a number of antiviral compounds, including zidovudine, lamivudine, efavirenz, indinavir, and the HIV-1 fusion inhibitor T-20, the latter against a panel of R5 HIV-1 isolates.³⁵ HIV-1 may escape from the above type of CCR5 antagonists, but such “escapes” do not involve switching in coreceptor use from CCR5 to CXCR4: instead, the escape mutant would seem able to continue to using CCR5 for entry into the cells despite the presence of the inhibitor.³⁶

Another class of low-molecular-weight CCR5 antagonists is represented by the spirodiketopiperazine E913 (**7**). E913 specifically blocked the binding of MIP-1 α to CCR5, the MIP-1 α -elicited cellular Ca²⁺



AMD3100

Scheme 8.

flux, and the replication of both laboratory and primary R5, as well as various multidrug-resistant monocyte/macrophage-tropic R5 HIV-1 strains.³⁷ From the spirodiketopiperazine class, AK602 was recently reported to bind only partially to CCR5, while exhibiting much greater anti-HIV activity; this compound was also quoted as blocking R5 HIV-1 replication in HIV-1 (JRFL)-infected hu-PBM NOD-SCID mice and to possess favorable oral bioavailability in rodents.³⁸

Almost simultaneously,^{39–41} three compounds, i.e., the bicyclam AMD3100,³⁹ [Tyr-5,12,Lys-7]polyphemusin or T22⁴⁰ and the nonapeptide (D-Arg)₉ or ALX40-4C⁴¹ were announced as CXCR4 antagonists, blocking the replication of T-tropic X4, but not M-tropic R5, HIV-1 strains through selective antagonism of CXCR4. The bicyclams are the most specific and most potent CXCR4 antagonists that have been described to date.^{42,43} The bicyclams had been known as potent and selective HIV inhibitors for a number of years,^{44,45} before their target of action was identified as the CXCR4 coreceptor.^{39,46,47} The bicyclam AMD3100 (**8**) inhibits the replication of X4 HIV-1 strains within the nanomolar concentration range.⁴⁵ As AMD3100 is not toxic to the host cells at concentrations up to 500 μ M, its selectivity index, or ratio of 50% cytotoxic concentration (CC_{50}) to 50% antivirally effective concentration (EC_{50}), can be estimated at $>100,000$.

A close correlation has been found, over a concentration range of 0.1–1000 ng/ml, between the AMD3100 concentrations required to inhibit

- (i) HIV-1 NL4-3 replication,
- (ii) monoclonal antibody (mAb 12G5) binding to the CXCR4 coreceptor, and
- (iii) SDF-1-induced signal transduction (Ca^{2+} flux), suggesting an intimate relationship between these three parameters.^{46,47}

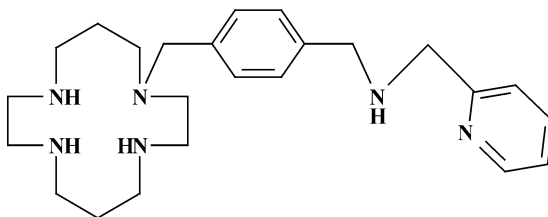
The inhibitory effects of AMD3100 on the T-tropic HIV-1 NL4-3 strain have been demonstrated in a wide variety of cells expressing CXCR4,

including peripheral blood mononuclear cells (PBMCs); and, *vice versa*, various T-tropic and dual-tropic, but not M-tropic, HIV-1 strains have proven sensitive to AMD3100 in PBMC.

Negatively charged amino acid (i.e., aspartic acid) residues in the extracellular regions of CXCR4 must be involved in its interaction with both AMD3100 and SDF-1, and the V3 loop of X4 HIV gp120, which are all three highly basic. Substitutions of a neutral amino acid residue for aspartic acid in the second extracellular loop generated resistance to AMD3100.⁴⁸ In particular, the aspartate residues at positions 171 and 262, located close to the extracellular sides of the transmembrane segments TM4 and TM6, may represent crucial sites of interaction with the bicyclam AMD3100.⁴⁹

The N-pyridinylmethylene cyclam AMD3465 (**9**), which, in contrast with the bicyclam AMD3100, contains only one monocyclam (1,4,8,11-tetraazacyclotetradecane) unit, is even 10-fold more effective as a CXCR4 antagonist, while showing no interaction whatsoever with CCR5. Mutational analysis identified His 281, in addition to Asp 171 and Asp 262 in CXCR4 as interaction sites for AMD3465. As expected, AMD3465 proved highly potent against X4 HIV strains but completely failed to inhibit the replication of R5 HIV-1 strains.⁵⁰

When the bicyclam AMD3100 was added to PBMC infected with clinical HIV isolates displaying the syncytium-inducing (SI) phenotype, these strains reverted to the non-syncytium-inducing (NSI) phenotype, and, concomitantly, these strains switched from CXCR4 to CCR5 coreceptor use.⁵¹ These findings indicate that selective blockade of CXCR4 by AMD3100 may prevent the switch from the less pathogenic M-tropic R5 to the more pathogenic T-tropic X4 strains of HIV, that *in vivo* heralds the progression to AIDS. AMD3100 has proved efficacious, alone and in combination with other anti-HIV drugs, in achieving a marked reduction in viral load in the SCID-hu Thy/Liv mouse model.⁵²



AMD3465

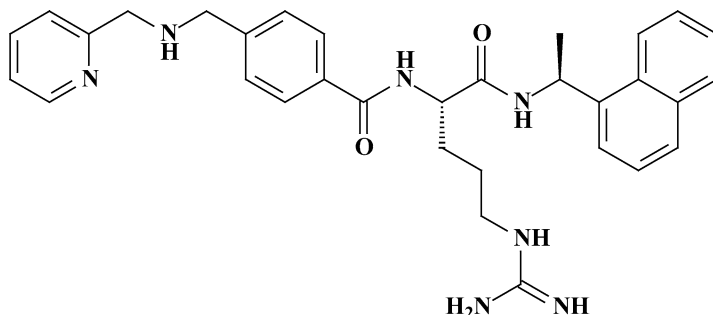
Scheme 9.

Following a phase I clinical trial for safety in normal healthy volunteers.⁵³ AMD3100 has recently entered phase II clinical trials in HIV-infected individuals, where it was found to effect a dose-dependent reduction of the T-tropic X4 HIV plasma viral load.⁵⁴ Notably, in 8 of 19 patients with dual (X4/R5) or mixed (X4 + R5) virus at baseline, a complete loss of X4 virus was observed by day 11 of treatment with an AMD3100 dose as low as 5 µg/(kg hour).⁵⁴ Thus, proof-of-principle has been provided that AMD3100 is able to suppress *in vivo* replication of X4 or X4/R5 HIV strains.⁵⁴ Earlier attempts with the nonapeptide ALX40-4C failed to show a significant or consistent reduction in viral load over a 1-month treatment period.⁵⁵

Given their high potency and selectivity as CXCR4 antagonists, bicyclams, such as AMD3100, may not only have great potential for the therapy and/or prophylaxis of X4 HIV infections, but also other pathologic (i.e. tumorigenic) processes, such as breast cancer metastasis, that are at least partially dependent of, or mediated by, signaling through CXCR4.⁵⁶ Also, AMD3100 may be pursued for its potential in the treatment of rheumatoid arthritis, since it was recently proven efficacious in suppressing collagen-induced arthritis (CIA) in interferon- γ receptor-deficient mice, an autoimmune joint disease that has been used as a model for the pathogenesis of rheumatoid arthritis in man.⁵⁷

Based on the (unexpected) observation during the phase I clinical studies that AMD3100 caused a significant enhancement of the white blood cell counts in human volunteers,⁵³ the compound was shown to mobilize hematopoietic stem cells from the bone marrow into the blood stream, and, as this effect is synergistic with the action of G-CSF (granulocyte-colony stimulating factor), AMD3100 is now pursued clinically (in phase II clinical studies) for stem cell mobilization and transplantation, i.e., in patients with multiple myeloma or non-Hodgkin lymphoma.

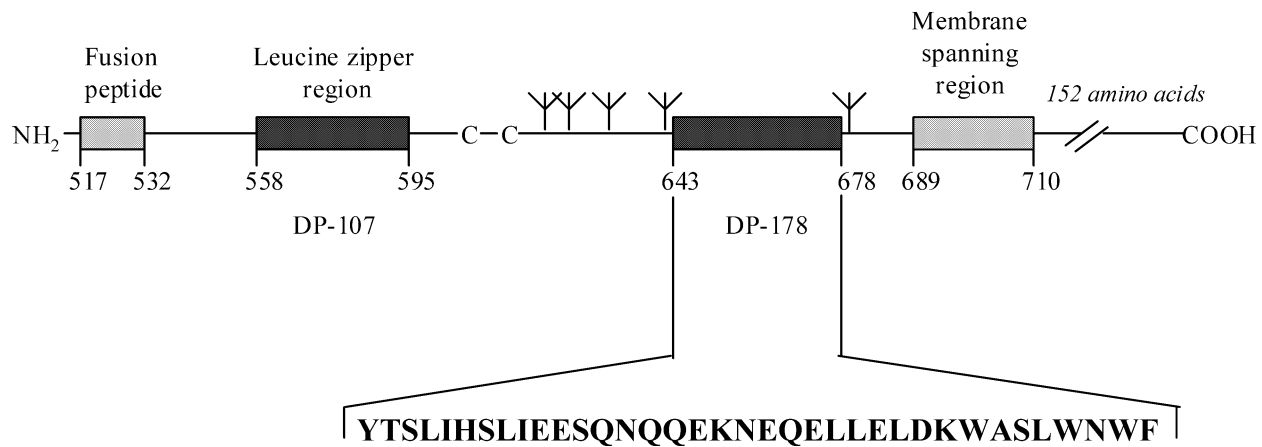
Recently, an orally bioavailable derivative of AMD3100, namely AMD070, was reported to exhibit an anti-HIV activity profile similar to that of AMD3100.⁵⁸ A phase I clinical study with AMD070 has been planned. Also, KRH-1636, another CXCR4 antagonist (**10**) seems to be endowed with an anti-HIV activity profile reminiscent of that of AMD3100; additionally KRH-1636 appears to be duodenally absorbable, at least in rats.⁵⁹

**KRH-1636***Scheme 10.*

IV. VIRAL FUSION (gp41) INHIBITORS

The interaction of the X4 or R5 HIV-1 envelope glycoprotein gp120 with the coreceptor CXCR4 or CCR5, respectively, is followed by a spring-loaded action of the viral glycoprotein gp41 (normally covered by the bulkier gp120), that then anchors through its amino terminus (the “fusion peptides”) into the target cell membrane. This initiates the fusion of the two lipid bilayers, that of the viral envelope with that of the cellular plasma membrane.⁶⁰ At the onset of the fusion process, the hydrophobic grooves on the surface of the N36 coiled coil in the gp41 ectodomain become available for binding with extraneous inhibitors, such as DP-178 (T-20), a 36-residue peptide, that binds to the hydrophobic groove of N36,⁶⁰ and N36^{Mut(e,g)} and N36^{Mut(a,d)}, two 36-residue peptides derived from the N36 peptide (residues 546–581 of the HIV-1 envelope).⁶¹

T-20 (Pentafuside, Enfuvirtide, Fuzeon) (**11**) is a synthetic, 36-amino acid peptide corresponding to residues 127–162 of the ectodomain of gp41 (or residues 643–678 in the gp160 precursor). T-20, previously called DP-178, was modeled after a specific domain (within gp41) predictive of α -helical secondary structure: DP-178 consistently afforded 100% blockade of virus-mediated cell–cell fusion (syncytium formation) at concentrations ranging from 1 to 10 ng/ml, i.e., 10^4 - to 10^5 -fold lower than the cytotoxic concentration.⁶² An initial clinical trial was carried out with T-20 at four doses (3, 10, 30 and 100 mg twice daily, intravenously, for 14 days) in sixteen HIV-infected adults: at the highest dose (100 mg, twice daily), T-20 achieved by the 15th day a 1.5- to 2.0-fold reduction in plasma HIV RNA.⁶³ These data provided proof-of-concept that HIV fusion inhibitors are able to reduce virus replication *in vivo*.



T-20 (Pentafuside, Enfuvirtide, Fuzeon)

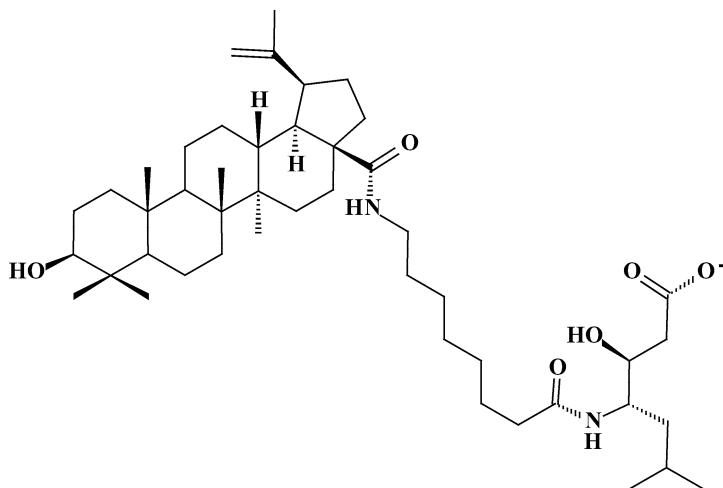
Scheme 11.

Meanwhile, T-20 has proceeded through phase III clinical trials (as a component of “salvage” therapy), and phase I clinical trials have been initiated with T-1249, a 39-amino acid peptide derived from DP-107 (which is a 38-amino acid peptide corresponding to residues 558–595 of gp160); T-1249 would be 10-fold more potent than T-20 when evaluated *in vitro* against HIV under the same conditions.⁶⁴

In a phase III, open-label, study, the so-called T-20 *versus* Optimized Regimen Only Study 1 (TORO 1),⁶⁵ patients from 48 sites in North and South America, with at least 5000 copies of HIV-1 RNA per ml of plasma were randomly assigned in a 2:1 ratio to receive enfuvirtide plus an optimized background regimen of three to five antiretroviral drugs or such a regimen alone (control group). Enfuvirtide (90 mg) was administered twice daily by subcutaneous injection. At 24 weeks, the mean change from base line in the viral load (intention-to-treat) was a decrease of $1.696 \log_{10}$ copies per ml in the enfuvirtide group, and a decrease of $0.764 \log_{10}$ copies per ml in the control group ($p < 0.001$). The mean increases in CD4⁺ cell count were 76 cells per μl and 32 cells per μl , respectively ($p < 0.001$). It was concluded that addition of enfuvirtide to an optimized antiretroviral drug regimen provided significant virologic and immunologic benefit through 24 weeks in patients who had previously received multiple antiretroviral drugs and had developed multi-drug HIV-1 resistance.⁶⁵ Enfuvirtide has been approved by the US FDA on 13 March 2003.

Sensitivity to T-20 is strongly influenced by coreceptor specificity: the EC₅₀ for R5 HIV-1 isolates was, at an average, $0.8 \log_{10}$ higher than the EC₅₀ for X4 HIV-1 isolates, this coreceptor specificity being defined by the gp120 V3 loop.⁶⁶ Baseline sensitivity to T-20 is determined by the N-terminal heptad repeat or first helical region (HR1) of gp41, where mutations G36D and V38A confer reduced sensitivity.^{66,67} These and other mutations seem to emerge promptly in patients upon monotherapy with T-20.⁶⁷ Given the variability in T-20 sensitivity between different virus strains depending on mutations in the HR1 domain of gp41, as well as modifications in the gp120 V3 loop that determine coreceptor specificity,⁶⁸ clinical use of fusion inhibitors such as T-20 may be made more efficient if combined with coreceptor antagonists.

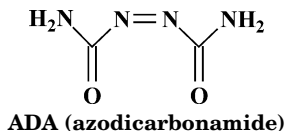
The betulinic acid derivative RPR 103611 (**12**) is the only non-peptidic low-molecular-weight compound that has been reported to block HIV-1 infection through interaction with gp41: this triterpene derivative has been found to inhibit the infectivity of a number of HIV-1 strains in the 10 nM concentration range,⁶⁹ apparently through interference with a post-binding, envelope-dependent step involved in the fusion of the virus with the cell plasma membrane. The exact mode

**RPR 103611***Scheme 12.*

of action of RPR 103611 remains to be elucidated. Sequence analysis of RPR103611-resistant mutants indicated that a single amino acid change, I84S, in HIV-1 gp41 is sufficient to confer drug resistance.⁷⁰ However, this I84S mutation did not occur in some of the naturally RPR103611-resistant HIV-1 strains such as NDK. More recently, the action of RPR103611 has been thought to depend on the accessibility of gp41,⁷¹ and for the isomeric betulinic acid derivative IC 9564, HIV-1 gp120, rather than gp41, has been proposed as the prime target (based on the mutations G237R and R252K emerging in gp120 of drug-resistant mutants).⁷² YK-FH312, a betulinic acid derivative unrelated to RPR103611 or IC 9564, was reported to block the assembly and/or budding of HIV particles.⁷³

V. NUCLEOCAPSID PROTEIN (NCp7) Zn FINGER-TARGETED AGENTS

The two zinc fingers (Cys-X₂-Cys-X₄-His-X₄-Cys (CCHC), whereby X = any amino acid) in the nucleocapsid (NCp7) protein⁷⁴ comprise the proposed molecular target for zinc-ejecting compounds such as NOBA (3-nitrosobenzamide), DIBA (2,2'-dithiobisbenzamide), SRR-SB3 (cyclic 2,2'-dithiobisbenzamide),⁷⁵ dithiane (1,2-dithiane-4,5-diol,1,1-dioxide)⁷⁶ and ADA (azodicarbonamide).^{77,78} These com-

*Scheme 13.*

pounds should be able to interfere with both early (uncoating, disassembly) and late phases (packaging, assembly) of retrovirus replication. Their effect at the early phase (disassembly) may also be ascribed to cross-linkage among adjacent zinc fingers. The DIBAs are able to enter intact virions, and the cross-linkage of NCp7 in virions correlates with loss of infectivity and decreased proviral DNA synthesis during acute infection.⁷⁹ Electron microscopically, the effect bestowed by DIBAs on virus morphology could be described as “core-freezing”.⁸⁰

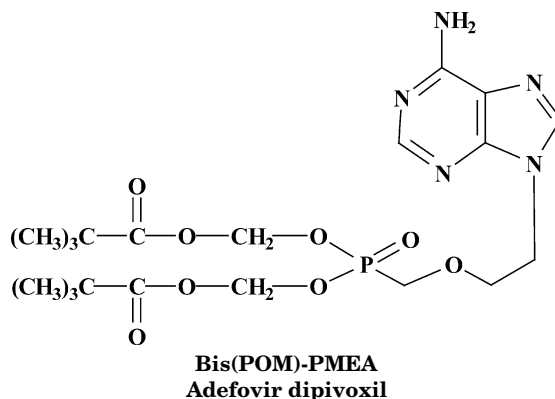
Although NOBA, DIBA, dithiane and ADA have been shown to dock nicely on the NCp7 Zn finger domains,⁸¹ and are believed to selectively target these Zn fingers without affecting the cellular Zn finger proteins, their selectivity indexes (ratio of CC₅₀ (50% cytotoxic concentration) over EC₅₀ (50% effective concentration)) are not that impressive.⁸¹ Of the NCp7-targeted compounds, ADA (**13**) has been the first to proceed to phase I/II clinical trials in advanced AIDS patients. Some preliminary evidence of efficacy was witnessed with add-on ADA in patients failing current antiretroviral therapy.⁸² These studies should be further extended. Although ADA is an HIV NCp7 Zn-finger inhibitor, its action *in vivo* is likely to be multipronged. ADA may well interact with a variety of targets, and, certainly its inhibitory effects on T-cell responses *in vitro* and *in vivo*⁸³ can hardly be attributed to an action targeted at the HIV NCp7 Zn fingers.

VI. REVERSE TRANSCRIPTASE (RT) INHIBITORS TARGETED AT THE SUBSTRATE-BINDING SITE

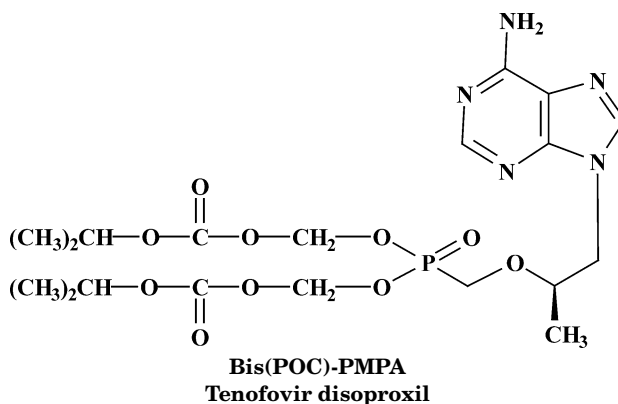
The substrate (dNTP) binding site of the HIV-1 RT is the target for a large variety of NRTI analogues, which have for several years⁸⁴ been recognized as efficacious drugs for the treatment of HIV infections: i.e., zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC), and the yet experimental drug emtricitabine [(-)FTC]. Fozivudine tidoxil is a thioether lipid zidovudine conjugate which has recently passed phase II clinical trials⁸⁵ and should be as effective as, and potentially better toler-

ated than, AZT. As a rule, all these compounds must be phosphorylated to their 5'-triphosphate form, before they can act as competitive inhibitors/substrate analogues/chain terminators at the reverse transcriptase level.

In contrast to the nucleoside analogues, the nucleotide analogues PMEa and PMPa (which can be termed NtRTIs) are already equipped with a phosphonate group, and therefore only need two phosphorylation steps to be converted to the active metabolite.⁸⁶ From PMEa and PMPa the oral prodrug forms [bis(POM)-PMEa or adefovir dipivoxil (**14**), and bis(POC)-PMPa or tenofovir disoproxil (**15**) fumarate, respectively] have been prepared. Adefovir dipivoxil has recently been



Scheme 14.



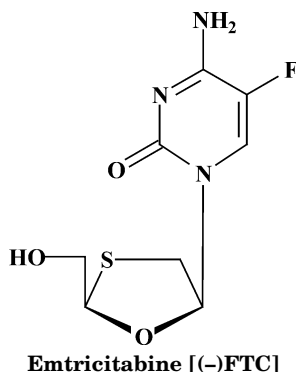
Scheme 15.

approved (September 2002 (US), March 2003 (EU)) for the treatment of chronic hepatitis B virus (HBV) infections; adefovir has demonstrated efficacy against 3TC-resistant HBV strains, while by itself not leading to resistance development, even after more than 2 years of treatment.

Tenofovir disoproxil fumarate (Viread[®]) has been recently approved for the treatment of HIV infections, after it proceeded swiftly through phase I/II⁸⁷ and phase III clinical trials, demonstrating that tenofovir (disoproxil fumarate) was well tolerated, that it reduced the viral load at non-toxic doses, and that it did virtually not induce any virus-drug resistance even after prolonged (> 1 year) treatment. In rhesus macaques infected with the highly pathogenic chimeric virus SHIV, tenofovir treatment initiated 1 week post infection, at a time when disseminated infection and extensive viral replication had already been established and CD4⁺ T-cell loss had begun, led to prompt, virtually complete suppression of viral replication and long-term stabilization of CD4⁺ T-cell levels, which were sustained, even after withdrawal of tenofovir (after 12 weeks of treatment).⁸⁸ In a monotherapy trial with tenofovir disoproxil fumarate in 10 chronically HIV-1-infected antiretroviral-naïve individuals, the drug achieved a 1.5 log₁₀ reduction in HIV-1 RNA levels during the 3 weeks of therapy, a response that was considered as robust as that observed with ritonavir monotherapy.⁸⁹

Apart from its efficacy, safety and tolerability, tenofovir disoproxil fumarate has a number of unique characteristics. It demonstrates activity against viruses resistant to the “classical” nucleoside analogues (i.e. zidovudine, didanosine and zalcitabine), including multidrug-resistant viruses carrying the Q151M mutation in their reverse transcriptase; and, furthermore, the long intracellular half-life of the active metabolite of tenofovir, its diphosphate PMPApp, which ranges from 12 to 15 hours in activated lymphocytes and from 33 to 50 hours in resting lymphocytes⁹⁰ allows infrequent, i.e., once-daily, dosing.

In a phase III, randomized, double-blind study of more than 552 antiretroviral therapy-experienced patients, given tenofovir disoproxil fumarate at 300 mg, once daily, for 48 weeks, the tenofovir-specific RT mutation K65R occurred in only 3% of the patients, and did not correlate with treatment failure; development of other NRTI mutations was due to the background drug regimen and did not appear to be associated with loss of HIV suppression.⁹¹ There was no evidence of MRP4 gene amplification [this multi-drug resistance protein belongs to the ATP-binding cassette (ABC), transporters that have been previously associated with adefovir resistance]. Preliminary clinical findings sug-



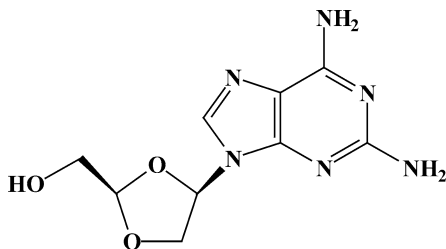
Scheme 16.

gest that, in comparison with stavudine, tenofovir would be less prone to lead to increased triglyceride or cholesterol levels (two parameters of lipid metabolism that in the long term are thought to be associated with lipodystrophy).^{92,93}

Of the various new nucleoside analogues (NRTIs), that have been recently described, and that encompass, among many others, (\pm)2'-deoxy-3'-oxa-4'-thiocytidine (BCH-10652, dOTC),⁹⁴ the dioxolane purine nucleoside analogues,⁹⁵ the methylenecyclopropane nucleoside analogues (and their phosphoro-L-alaninate diesters)^{96,97} and the 4'-ethynyl nucleoside analogues,⁹⁸ the most advanced in clinical development is emtricitabine (2', 3'-dideoxy-3'-thia-5-fluorocytidine, (-)FTC).

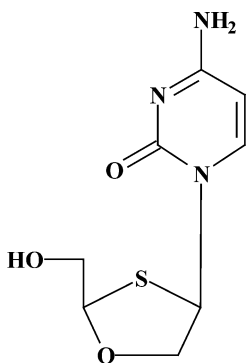
Emtricitabine [(-)FTC] (**16**) has been chosen at a once-daily dose of 200 mg for long-term clinical studies in HIV-1-infected subjects.⁹⁹ A once-daily combination therapy of emtricitabine with didanosine and efavirenz has proven safe and both antivirally and immunologically effective for at least 24 weeks.¹⁰⁰ Emtricitabine has been considered an ideal drug candidate¹⁰¹ in that it shows synergism with other antiretrovirals, excellent tolerability, a long intracellular half-life supportive of once-daily dosing, and, in comparison with lamivudine, 4- to 10-fold higher *in vitro* potency against HIV.

Amdoxovir [DAPD, (-)- β -D-2,6-diaminopurine dioxolane] (**17**) has *in vitro* activity against both HIV and HBV; it is converted by adenosine deaminase to dioxolane guanine (DXG), which is then phosphorylated intracellularly to DXG 5'-triphosphate, the active metabolite.¹⁰² DAPD/DXG has proven active against AZT- and 3TC-resistant HIV-1 strains,^{102,103} but has decreased activity against viruses containing the K65R and Q151M mutations.¹⁰⁴ An attempt has been made to explain in molecular terms the activity of DAPD/DXG against AZT- and



Amdoxovir
 (-)- β -D-2,6-Diaminopurine dioxolane (DAPD)

Scheme 17.

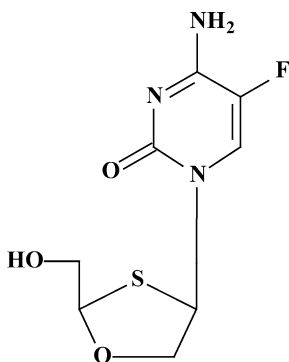


(±)2'-Deoxy-3'-oxa-4'-thiocytidine (dOTC)

Scheme 18.

3TC-resistant HIV-1 strains.¹⁰⁵ Amdoxovir has proceeded to phase I/II clinical studies. The antiviral activity of DAPD has been demonstrated in the human peripheral blood mononuclear cell-severe combined immunodeficiency (hu PBMC-SCID) mouse model, in which virus levels were reduced in peritoneal cells, lymph nodes, and splenocytes.¹⁰⁶ As has been demonstrated for combinations of mycophenolic acid with the antiherpetic guanosine analogues acyclovir, ganciclovir, and penciclovir, mycophenolic acid also enhanced the anti-HIV activity of DAPD, by decreasing the intracellular dGTP levels, consequently to its inhibitory effect on IMP dehydrogenase.¹⁰⁴

BCH-10652 (dOTC) (**18**) has demonstrated activity against HIV-1 in the SCID-hu Thy/Liv model. Despite its structural similarity to 3TC, dOTC proved also active against 3TC-resistant HIV-1 (M184V), albeit at a relatively high dosage level (400 mg/(kg day)).¹⁰⁷ Also *in vitro*,



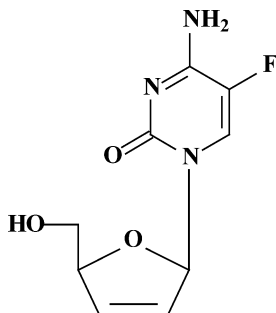
(±)2'-Deoxy-3'-oxa-4'-thiocytidine (FdOTC)
Racivir[®] (RCV)

Scheme 19.

dOTC and its (+) and (–) enantiomers, still retained, albeit reduced, activity against 3TC-resistant M184V and M184I HIV-1 mutants.¹⁰⁸

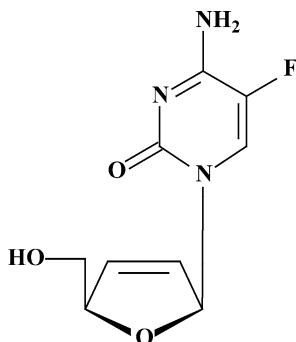
Racivir (RCV) (**19**) corresponds to 5-fluoro-substituted dOTC: as suggested by its name, it is racemic, consisting of both the (+)- and (–)-enantiomeric forms (whereby the (+) form would be 9-fold less active than, but synergistic with the (–) form). RCV has potent and selective activity against HIV and HBV *in vitro* and in animal models;¹⁰⁹ it has excellent oral bioavailability and has already been the subject of a phase I/II clinical study in combination with stavudine and efavirenz; this combination was well tolerated and effected a more than 2 log₁₀ decrease in viral load.¹⁰⁹ RCV may be useful as a once-a-day component at a dose that still needs to be defined (200, 400 or 600 mg daily).

Reverset (RVT, DPC 817, β-D-d4FC) (**20**) corresponds to the 5-fluoro-substituted derivative of d4C; d4C was already described in 1986 as a potent and selective anti-HIV agent.¹¹⁰ D-d4FC retains activity against HIV-1 isolates harboring mutations in the reverse transcriptase gene that confer resistance to 3TC or AZT;¹¹¹ however, it seems less potent against multi-NRTI-resistant viruses, particularly those carrying the Q151M mutation.¹¹² In addition, RVT itself selects for the RT K65R mutation which confers 5.3- to 8.7-fold resistance to RVT *in vitro*.¹¹² Reverset (β-D-d4FC) is readily converted to its triphosphate in human peripheral blood mononuclear cells and interacts synergistically with a number of other anti-HIV agents.¹¹³ A phase I clinical study has demonstrated that the desired plasma concentrations of RVT can be easily achieved with an oral dose of 50 mg; RVT may be use-



Reverset (RVT, DPC 817)
 β -D-2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine (β -D-d4FC)

Scheme 20.

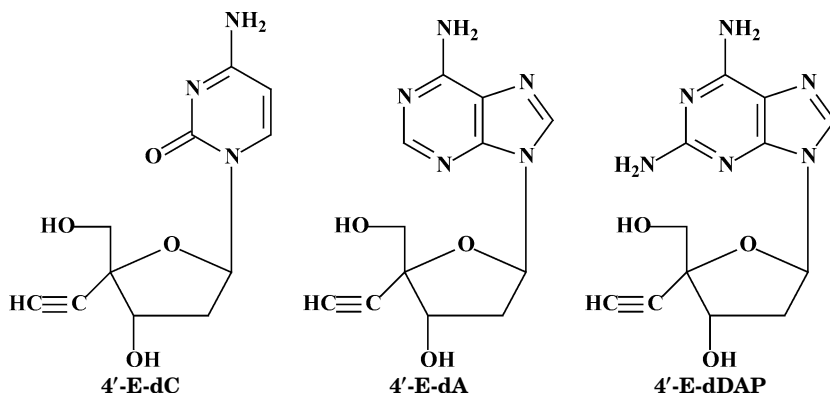


Elvucitabine (ACH-126,443)
 β -L-2',3'-didehydro-2',3'-dideoxy-5-fluorocytidine (β -L-d4FC)

Scheme 21.

ful as a once-a-day component for the treatment of NRTI-experienced patients.¹¹⁴ In the SCID-hu Thy/LIV mouse model RVT effected a viral load reduction below the detection limit for both NL4-3 and 3TC-resistant M184V HIV-1 strains.¹¹⁵

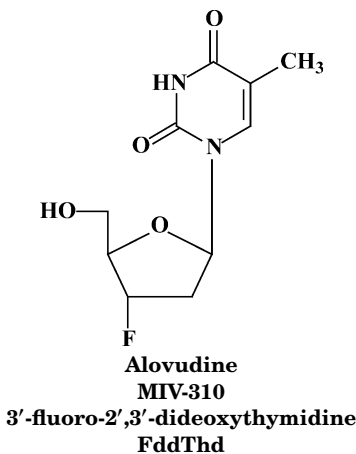
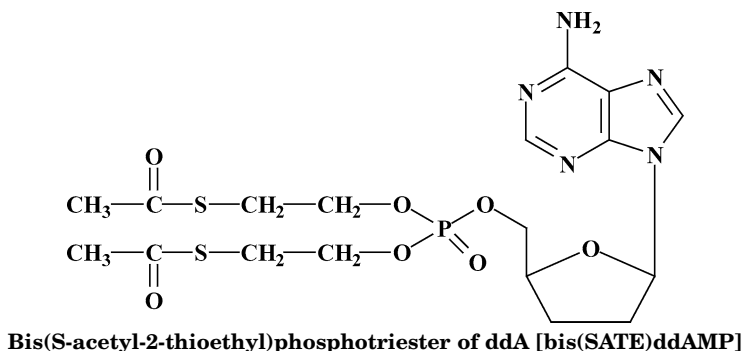
Elvucitabine (ACH-126,443, β -L-d4FC) is the L-counterpart of β -D-d4FC (Reverset). β -L-d4FC (**21**) has potent *in vitro* activity against both HIV and HBV. Its EC_{50} against HIV-1 is 0.3 ng/ml, that is 100-fold lower than that for 3TC.¹¹⁶ An ongoing phase I/II clinical trial has seemingly demonstrated that elvucitabine may be efficacious in NRTI-experienced patients in reducing the viral load when administered orally once daily at doses of 50 and 100 mg.¹¹⁶

*Scheme 22.*

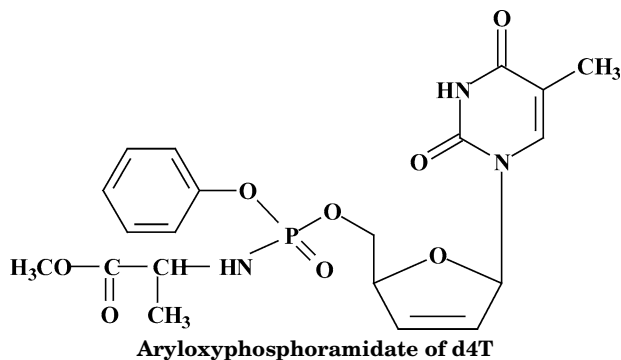
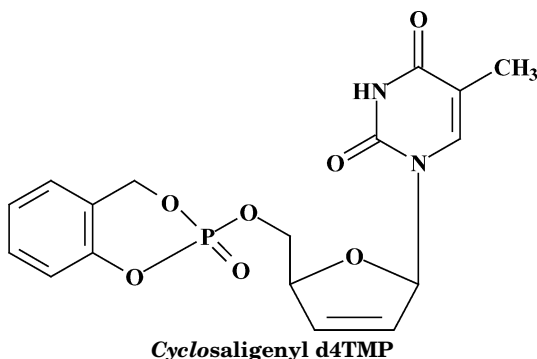
The 4'-ethynyl-2'-deoxynucleosides (4'-E-dNs)⁹⁸ represent a new family of NRTIs which, despite the presence of a 3'-hydroxyl group, appear to function as viral DNA chain terminators.¹¹⁷ Among the 4'-E-dNs, the cytosine, adenine and 2,6-diaminopurine derivatives (**22**), termed 4'-E-dC, 4'-E-dA and 4'-E-dDAP, exert potent activity against a variety of HIV-1 strains, including AZT-resistant (M41L/T215Y and M41L/T69SSG/T215Y) strains, 3TC-resistant (M184V and M184I) strains and multidideoxynucleoside-resistant (MDR) strains (containing the hallmark Q151M mutation for MDR).¹¹⁷ The mutations that HIV-1 developed in response to the 4'-E-dNs were T165I and M184I (thus far), but these mutations did not lead to a significant decrease in the sensitivity to the 4'-E-dNs.¹¹⁷ Although extremely potent and endowed with high selectivity indexes, the 4'-E-dNs might, because of toxicity reasons, accomplish only limited clinical usefulness.

From the initial studies on the anti-HIV-1 activity of a large variety of 2',3'-dideoxynucleoside analogues, the 3'-fluoro-2',3'-dideoxythymidine (FddThd) emerged as the most potent, but also the most cytotoxic, anti-HIV compound, with an EC₅₀ and CC₅₀ of 0.001 and 0.197 μ M, respectively (selectivity index: 197).¹¹⁸ It is intriguing that this compound (also termed MIV-310 (Alovudine) (**23**)) has now re-emerged as a potentially clinically useful agent: in a pilot 4-week phase II study in patients failing multiple antiretroviral therapy, MIV-310 at a once-daily oral dose of 7.5 mg, added onto the ongoing multidrug therapy, brought about a significant reduction in viral load without serious adverse events.¹¹⁹

The bottleneck in the metabolic pathway leading from AZT and the other 2',3'-dideoxynucleoside (ddN) analogues to their active 5'-

*Scheme 23.**Scheme 24.*

triphosphate form is the first phosphorylation step. Therefore attempts have been made at constructing 2',3'-dideoxynucleotide (ddNMP) prodrugs, that, once taken up by the cells, deliver the nucleotide (ddNMP) form. This approach has proven particularly successful for a number of NRTIs such as 2',3'-dideoxyadenosine (ddA) and d4T. Thus, the bis(S-acetyl-2-thioethyl)phosphotriester of ddA [bis(SATE)ddAMP] (**24**) was synthesized and found to be 1000-fold more potent against HIV than the parent compound ddA.¹²⁰ Similarly, aryloxyphosphoramidate derivatives of d4T (i.e., So324, a d4T-MP prodrug containing at the phosphate moiety a phenyl group and the methylester of alanine linked to the phosphate group through a phosphoramidate

*Scheme 25.**Scheme 26.*

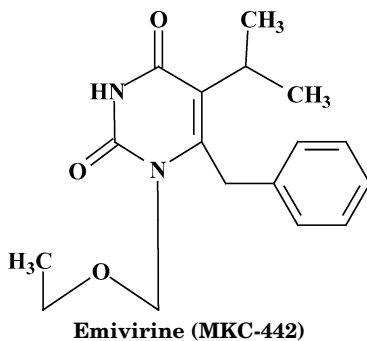
linkage) have been constructed.^{121–123} After the d4T aryloxyphosphoramidate (**25**) has been taken up by the cells, d4TMP is released intracellularly and then processed onto its active metabolite d4TTP.¹²⁴ This “thymidine kinase bypass” explains the high anti-HIV activity of d4T aryloxyphosphoramidate derivatives in thymidine kinase deficient cells and resting monocytes/macrophages.¹²⁵ The thymidine kinase (in the case of d4T) and the adenosine deaminase (in the case of ddA) can also be bypassed by using the cyclic saligenyl approach.^{126,127} *CycloSaligenyl* pronucleotides of d4T and ddA deliver exclusively the nucleotides d4TMP and ddAMP, not only under chemical-simulated hydrolysis conditions but also under intracellular conditions.^{128,129} This has been convincingly shown for the *cyclosaligenyl* derivative of d4TMP (**26**) in a number of cell lines.¹³⁰

VII. REVERSE TRANSCRIPTASE INHIBITORS TARGETED AT AN ALLOSTERIC, NON-SUBSTRATE-BINDING SITE

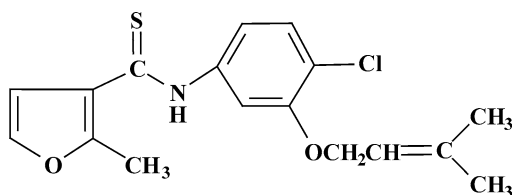
More than 30 structurally different classes of compounds have been identified as NNRTIs, *viz.* compounds that are specifically inhibitory to HIV-1 replication and targeted at a non-substrate binding site of the reverse transcriptase.¹³¹ Three NNRTIs (nevirapine, delavirdine and efavirenz) have so far been formally licensed for clinical use in the treatment of HIV-1 infections. Emivirine (MKC-442) (**27**) has been the subject of advanced (phase III) clinical trials, but was then discontinued for further development, in spite of preclinical safety and pharmacokinetics assessments that supported the continued development, as a treatment for HIV-1 infection, in large-scale clinical trials with adults as well as pilot studies with children and pregnant women.¹³² Closely related structural analogues of emivirine, *i.e.*, with a thiocyclohexyl or 3,5-dimethylbenzyl substituted for the benzyl group, showed a 30-fold greater inhibitory activity than emivirine against the K103N and Y181C reverse transcriptase mutant strains.¹³³

The NNRTIs interact with a specific “pocket” site of the HIV-1 RT,¹³⁴ which is closely associated with, but distinct from, the substrate binding site. NNRTIs are notorious for rapidly eliciting resistance,¹³⁵ resulting from mutations at the amino acid residues that surround the NNRTI-binding site of HIV-1 RT. However, emergence of NNRTI-resistant HIV strains can be prevented if the NNRTIs are combined with NRTIs and used from the beginning at sufficiently high concentrations.¹³¹

The thiocarboxanilide UC-781 (**28**) is an exceptionally potent inhibitor of HIV-1 replication.¹³¹ It has been found to restore the antiviral activity of AZT against AZT-resistant HIV-1.¹³⁶ UC-781 has been



Scheme 27.



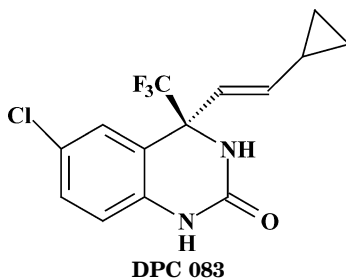
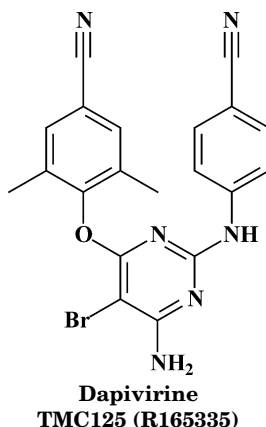
Thiocarboxanilide UC-781

Scheme 28.

recognized as a (retro)virucidal agent, capable of reducing the infectivity of HIV-1 virions, and, therefore, yielding considerable promise for the use in (retro)virucidal formulations to prevent the transmission of HIV from infected to noninfected individuals.¹³⁷ UC-781 would seem an ideal candidate for application as a vaginal microbicide (virucide), i.e., when formulated in replens gel.¹³⁸

To the new classes of NNRTIs that offer potent anti-HIV-1 activity belong the thieno[3,4-*c*][1,2,4]thiadiazine derivative QM96521,¹³⁹ the quinoxaline GW420867X,¹⁴⁰ the imidazole derivative S-1153 (AG1549, capravirine),^{141–143} (–)-6-chloro-2-[(1-furo[2,3-*c*]pyridin-5-yl)ethylthio]-4-pyrimidinamine (PNU-142721),¹⁴⁴ N-[2-(2,5-dimethoxyphenylethyl)-N'-[2-(5-bromopyridyl)]thiourea (HI-236),¹⁴⁵ the pyrido[1,2-*a*]indole derivative BCH-1,¹⁴⁶ the 4-cyclopropylalkenyl-4-trifluoromethyl-3,4-dihydro-2(1*H*)quinazolinones DPC 082 and DPC 083,¹⁴⁷ the thiophene-ethylthiourea (TET) derivative HI-443,¹⁴⁸ the cyclohexenylethylthiourea derivatives HI-346 and HI-445,¹⁴⁹ the *cis*-cyclopropyl urea-PETT derivatives,¹⁵⁰ the alkenyldiarylmethane (ADAM) series of compounds,¹⁵¹ the pyrrolobenzoxazepinone (PBO) derivatives,¹⁵² the quinoxalinyethylpyridyl thioureas (QXPTs),¹⁵³ the emivirine (MKC-442) derivative SJ-3366,¹⁵⁴ the 3,4-dihydro-2-alkoxy-6-benzyl-4-oxypyrimidines (DABOs)¹⁵⁵ and the dianilinopyrimidine (DAPY) derivatives (i.e., TMC125).¹⁵⁶

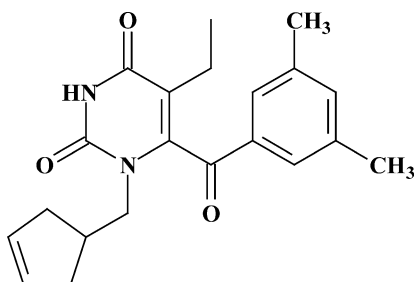
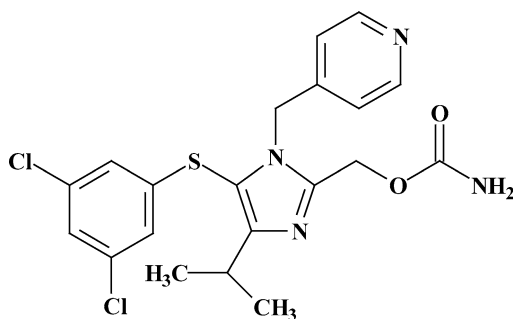
As a rule, the “new” (“second” generation) NNRTIs exhibit higher potency than the “old” (“first” generation) NNRTIs against wild-type and NNRTI-resistant HIV-1. This is particularly prominent for DPC 083 (**29**) and TMC125 (**30**) that showed activity against L100I, K103N, Y181C, Y188L, K103N + L100I and K103N + Y181C reverse transcriptase mutant strains in the nanomolar concentration range.^{147,156} This makes TMC125 and DPC 083 excellent candidates for further clinical development. Because of their potent activity against high multiplicities of infection (MOIs), DABOs (such as MC 1220)¹⁵⁵ and DAPYs (i.e.,

*Scheme 29.**Scheme 30.*

TMC120) also yield great potential as microbicides to prevent mucosal HIV transmission.

When administered orally twice daily at a dose of 900 mg for 7 days in treatment-experienced patients with highly NNRTI-resistant virus and currently failing on an NNRTI-containing regimen, TMC125 demonstrated significant antiviral potency (viral load reduction up to $0.9 \log_{10}$).¹⁵⁷ In another study, monotherapy with TMC125 in antiretroviral-naïve HIV-1-infected individuals effected, after 1 week of treatment, a $1.5\text{--}2.0 \log_{10}$ reduction of viral load, with a similar initial rate of decline of plasma HIV-1 as seen with a 5-drug regimen.¹⁵⁸

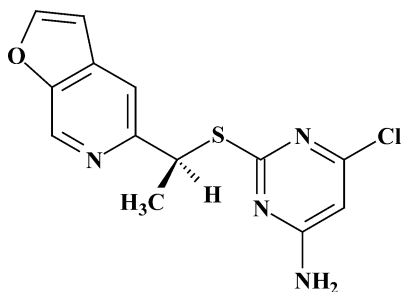
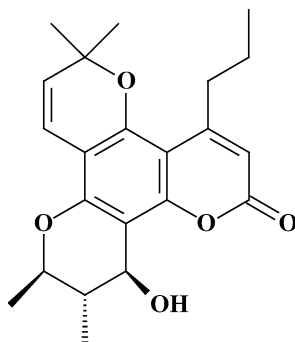
DPC 083, administered orally once daily at a dose of 100 mg, in patients who had failed on the current NNRTIs and harbored NNRTI-resistant mutations, effected, after 8 weeks of treatment, a viral load reduction of $1.28 \log_{10}$.¹⁵⁹ In antiretroviral-naïve HIV-1-infected indi-

**SJ-3366***Scheme 31.***Capravirine (S-1153, AG1549)***Scheme 32.*

viduals, DPC 083 at an oral once-daily drug regimen of 50, 100 or 200 mg provided trough plasma drug levels that exceeded the IC_{90} for NNRTI-resistant HIV-1 mutants.¹⁶⁰

Some of the new NNRTIs, such as SJ-3366 (**31**), possess remarkable features. This compound was reported to inhibit HIV-1 replication at a concentration below 1 nM with a therapeutic index greater than 4,000,000, and to inhibit HIV-2 replication (albeit at higher concentrations than those required for inhibition of HIV-1) at the viral entry stage.¹⁵⁴

Capravirine (AG1549) (**32**) has a favorable profile of resilience to many drug resistance mutations, which has been attributed to extensive main chain hydrogen bonding involving the main chain of residues K101, K103, and P236 of the p66 reverse transcriptase subunit.¹⁴² In particular, the activity of capravirine against HIV-1 strains carrying the clinically relevant K103N mutation is noteworthy. Capravirine has proceeded to phase II/III clinical trials,¹⁴³ which were temporarily put

**PNU-142721***Scheme 33.***(+)-Calanolide A***Scheme 34.*

on hold (due to animal toxicity studies showing vasculitis in dogs after 48 weeks of drug administration), but have now been resumed.

The NNRTIs *cis*-cyclopropylurea-PETT¹⁵⁰ and pyrrolobenzoxazepinone (PBO) derivatives¹⁵² are orally bioavailable and penetrate well into the brain. The broad, potent antiviral activity and favorable pharmacokinetic profile, have led to the selection of PNU-142721 (**33**) for clinical studies;¹⁴⁴ and DPC 082 and DPC 083 for clinical development.¹⁴⁷

(+)-Calanolide A (**34**) is the only naturally occurring NNRTI. It was first isolated from a tropical tree (*Calophyllum lanigerum*) and has already been the subject of a phase I clinical study in healthy, HIV-negative individuals.¹⁶¹

Recently, an unexpected effect of NNRTIs on HIV-1 RT dimerization was documented:¹⁶² several NNRTIs, including efavirenz, were found to enhance the association between the RT subunits p66 and p51, apparently due to a conformational change in the p66 subunit that re-

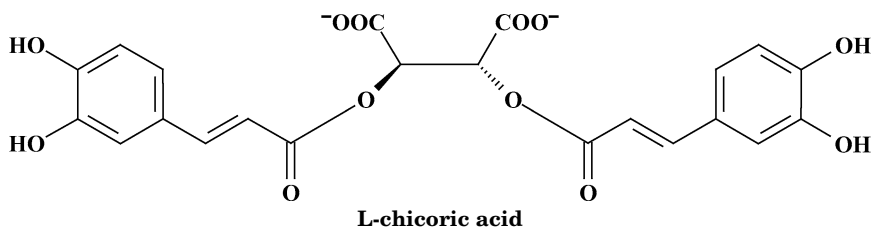
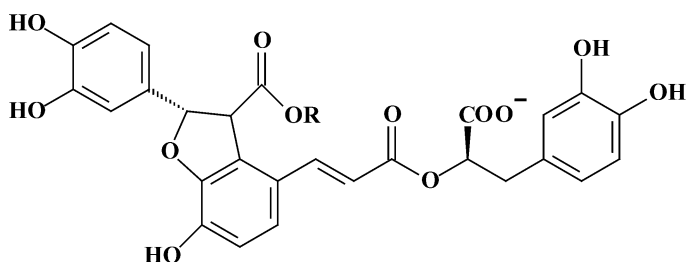
sulted in enhanced binding to the p51 subunit. It remains to be established if this enhanced dimerization has any bearing on the anti-HIV-1 potency of the NNRTIs.

The dimerization of the RT subunits p66 and p51 may be considered as a potential target for anti-HIV agents. This dimerization can be blocked by synthetic oligopeptides corresponding to the amino acids 395–404 of the HIV RT (i.e., (oligo)peptide no 7: KEIWEIWWIE) or extended versions thereof (i.e., (oligo)peptide no 1: FKLPIQKETWETWWTEYWE).¹⁶³

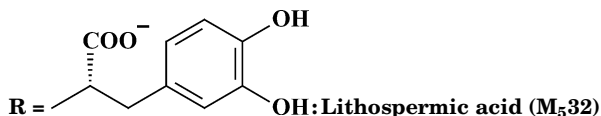
VIII. HIV INTEGRASE INHIBITORS

Retrovirus integration requires at least two viral components, the retroviral enzyme integrase, and *cis*-acting sequences at the retroviral DNA termini U3 and U5 ends of the long terminal repeats (LTRs). Since HIV, like other retroviruses, cannot replicate without integration into a host chromosome, integrase has been considered as an attractive therapeutic target. Numerous compounds have been described as inhibitors of HIV-1 integrase (for a recent review, see Pommier et al.¹⁶⁴): for example, polyamides, bisdistamycins and lexitropsins,¹⁶⁵ polyhydroxylated aromatic type of compounds, including ellagic acid, purpurogallin, 4,8,12-trioxatricornan and hypericin,¹⁶⁶ and a series of thiazolothiazepine derivatives, preferably possessing the pentatomic moiety SC(O)CNC(O) with two carbonyl groups.¹⁶⁷ The problem with integrase inhibitors is that, while they might be effective in an enzyme-based assay, their anti-HIV activity in cell culture may be masked by cytotoxicity, and if they do exhibit anti-HIV activity, this could, at least in some cases, be attributed to antiviral actions targeted at other steps in the HIV replicative cycle.

L-chicoric acid^{168–170} is such a case. L-chicoric acid is structurally reminiscent of curcumin,¹⁷¹ 3,5-dicaffeoylquinic acid,¹⁷² rosmarinic acid¹⁷³ and dicaffeoyltartaric acids (DCTAs),¹⁷⁴ and all these compounds have been reported to inhibit HIV-1 integrase. Integrase was identified as the molecular target for the action of L-chicoric acid (**35**), since a single amino acid substitution (G140S) in the integrase rendered the corresponding HIV-1 mutant resistant to L-chicoric acid.¹⁷⁰ We have recently demonstrated,¹⁷⁵ however, that L-chicoric acid owes its anti-HIV activity in cell-culture to an interaction with the viral envelope gp120. Upon repeated passages of the virus in the presence of the compound, mutations were found in the V2, V3 and V4 loop of

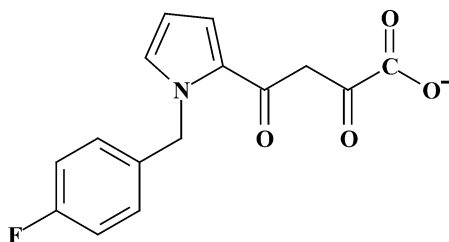
*Scheme 35.*

R = H: Lithospermic acid (M₅22)

*Scheme 36.*

gp120, while no mutations were seen in the integrase. We did confirm that in an enzymatic assay L-chicoric acid inhibited HIV integrase activity, but integrase carrying the G140S mutation appeared to be as sensitive to the inhibitory effect of L-chicoric acid as the wild-type integrase. Furthermore, L-chicoric acid proved inactive against HIV strains that were resistant to polyanionic compounds known to interact at the virus adsorption level, and time-of-addition experiments further corroborated an interaction of L-chicoric acid at the virus adsorption stage.¹⁷⁵

From the herbal plant, *Salvia miltiorrhiza* roots, two components were isolated, namely lithospermic acid (M₅22) and lithospermic acid B (M₅32) (**36**), that were claimed to be genuine, non-toxic inhibitors of HIV-1 integrase, not affecting HIV entry.¹⁷⁶ Curiously, M₅32 was more effective than M₅22 in inhibiting the 3'-processing of HIV-1 integrase (IC₅₀: 0.48 and 0.83 μ M, respectively), whereas, conversely, M₅22 was

**L-731,988****Scheme 37.**

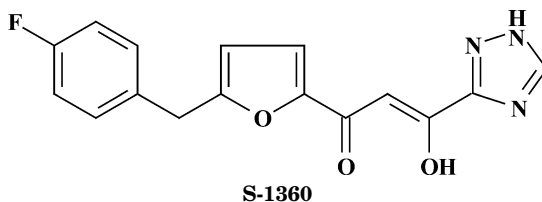
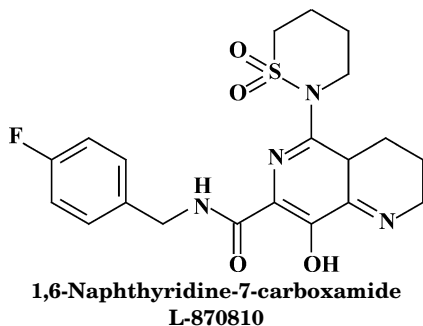
more efficacious than M₅32 in inhibiting virus replication (EC₅₀: 2 and 6.9 μ M, respectively).¹⁷⁶

The structure of the HIV-1 integrase core domain complexed with the inhibitor 5CITEP (1-(5-chloroindol-3-yl)-3-hydroxy-3-(2*H*-tetrazol-5-yl)-propenone) has been described as a platform for structure-based design of novel HIV-1 integrase inhibitors.¹⁷⁷ This was followed by the description of a number of diketo acids (such as L-731,988 (**37**) and L-708,906) as inhibitors of the integrase-mediated strand transfer reaction that leads to the covalent linkage of the viral DNA 3' ends to the cellular (target) DNA.¹⁷⁸ Starting from L-731,988, additional 4-aryl-2,4-dioxobutanoic acid derivatives have been reported as inhibitors of HIV-1 integrase.¹⁷⁹

L-731,988 and L-708,906 were also found to inhibit HIV-1 replication in cell culture. In cell culture, addition of L-708,906 could be postponed (without loss of activity) for 7 hours after infection, a time point that coincides with proviral DNA integration. Inhibition of integration in cell culture was confirmed by quantitative *Alu*-PCR.¹⁸⁰

Furthermore, mutations in the HIV-1 integrase were found to confer resistance to the inhibitory effects of L-731,988 on both strand transfer and HIV-1 infectivity.¹⁷⁸ Thus it was surmised that the diketo acids owe their antiviral activity exclusively to inhibition of one of the two catalytic functions of integrase, namely strand transfer (the other catalytic function being endonucleolytic processing of the (pro)viral DNA to remove the terminal dinucleotide (GT) from the 3' end).¹⁷⁸

Antiviral resistance to the diketo acid L-708,906 is associated with the mutations T66I, L74M and S230R in the HIV-1 integrase gene:¹⁸¹ after 30 passages of HIV-1 in the presence of L-708,906, the T66I mutant was detected. On further passaging (up to 70 passages), the virus retained the T66I mutation but, in addition, acquired the L74M and S230R mutations. This multiple-mutant virus proved highly resistant to the diketo acid L-708,906.¹⁸¹

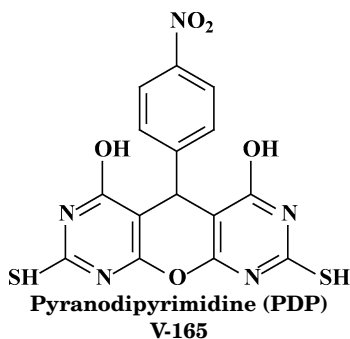
*Scheme 38.**Scheme 39.*

Reportedly in clinical development is the diketo derivative S-1360 (**38**): this compound was recently announced as an HIV-1 integrase inhibitor for oral use.¹⁸² S-1360 would inhibit the HIV-1 integrase at an IC_{50} of 20 nM, and HIV-1 replication at an EC_{50} of 140 nM, while its CC_{50} would be 110 μ M, thus achieving a therapeutic index of almost one thousand.¹⁸²

Another HIV-1 integrase inhibitor that reportedly entered clinical development is the 1,6-naphthyridine-7-carboxamide (**39**) L-870810.¹⁸³

The mechanism of action of the diketo acids (i.e., L-731,988), as well as 5CITEP, S-1360 and L-870810 may be based on an interaction between the carboxylate of the diketo acid or the isosteric heterocycle in the other compounds and metal ion(s) in the active site of the integrase, resulting in a functional sequestration of these critical metal cofactors.¹⁸⁴

Recently, an entirely new class of HIV integrase inhibitors was identified, namely that of the 5*H*-pyrano[2,3-di:-6,5-d']dipyrimidines (PDPs); the most potent congener of this series, 5-(4-nitrophenyl)-2,8-dithiol-4,6-dihydroxy-5*H*-pyrano[2,3-d:-6,5-d']dipyrimidine (V-165) (**40**) inhibited the replication of HIV-1 at an EC_{50} of 8.9 μ M, which is 14-fold below the cytotoxic threshold.¹⁸⁵ A close correlation was

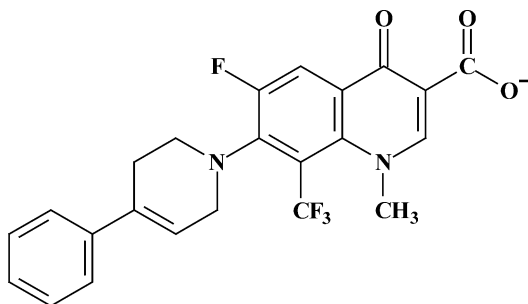
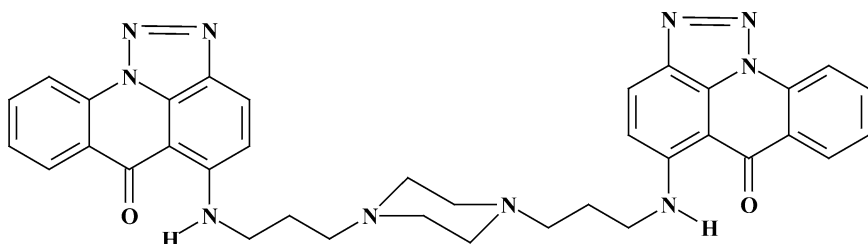
*Scheme 40.*

found between the anti-HIV activity observed in cell culture and the inhibitory activity in the integrase strand transfer assays. Time-of-addition experiments confirmed that V-165 interfered with the viral replication cycle at a time point coinciding with integration, a conclusion corroborated by *Alu*-PCR.¹⁸⁵

IX. TRANSCRIPTION (TRANSACTIVATION) INHIBITORS

At the transcription level, HIV gene expression may be inhibited by compounds that interact with cellular factors that bind to the LTR promoter and that are needed for basal level transcription, such as the NF- κ B inhibitors.¹⁸⁶ Greater specificity, however, can be expected from those compounds that specifically inhibit the transactivation of the HIV LTR promoter by the viral Tat (*trans*-activating) protein.¹⁸⁶ Tat has pleiotropic effects: it not only activates the transcription of HIV-1 RNA, but also binds to a number of receptors, i.e., on smooth muscle and skeletal muscle cells;¹⁸⁷ the basic domain of Tat may be important, not only for translocation but also for nuclear localization and *trans*-activation, and thus targeting of the Tat basic domain may provide great scope for therapeutic intervention in HIV-1 infection.¹⁸⁷

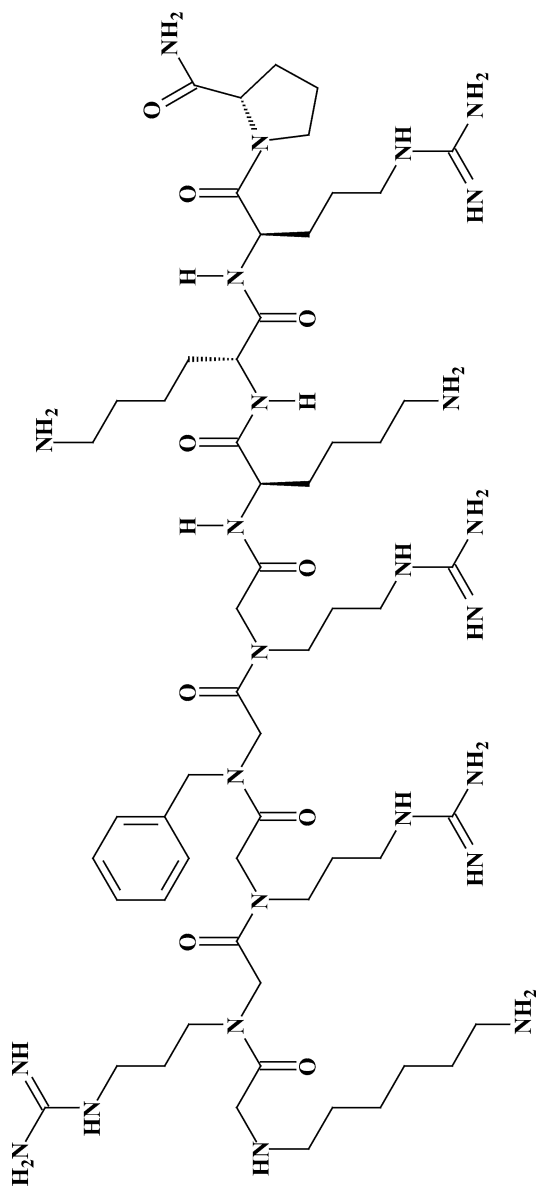
A number of compounds have been reported to inhibit HIV-1 replication in both acutely and chronically infected cells through interference with the transcription process: i.e., fluoroquinoline derivatives.¹⁸⁸ The inhibitory effects of the fluoroquinolines (K-12) [8-difluoromethoxy-1-ethyl-6-fluoro-1,4-dihydro-7-[4-(2-methoxyphenyl)-1-piperazinyl]-4-oxoquinoline-3-carboxylic acid] and (K-37) [7-(3,4-dehydro-4-phenyl-1-piperidiny)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid] (**41**) on the HIV-1 LTR-driven gene expres-

**Fluoroquinoline K-37***Scheme 41.***Temacrazine***Scheme 42.*

sion may at least in part be attributed to inhibition of Tat¹⁸⁹ or other RNA-dependent transactivators.¹⁹⁰

The bistriazoloacridone temacrazine [1,4-bis(3-(6-oxo-6H-v-triazolo-[4,5,1-de]acridin-5-yl-aminopropyl)piperazine)] (**42**) was found to block HIV-1 RNA transcription from the HIV proviral DNA without interfering with the transcription of any cellular genes.¹⁹¹ The compound inhibited HIV-1 replication in both acutely and chronically infected cells. Resistance was generated upon serial passage of the virus in the presence of temacrazine and was associated with several unique nucleotide changes in HIV-1 LTR at positions -1, -2 and +111 relative to the start of transcription.¹⁹²

Tat peptide analogs, encompassing the Tat core domain (amino acid residues 36–50),¹⁹³ or the basic domain (amino acids 48–56: RKKRRQRRR)¹⁹⁴ have been reported to inhibit HIV-1 replication, and, as expected, these peptide analogs were able to effectively block the Tat transactivation process. The 9-mer peptoid CGP64222 (**43**), which is structurally reminiscent of the amino acid 48–56 sequence



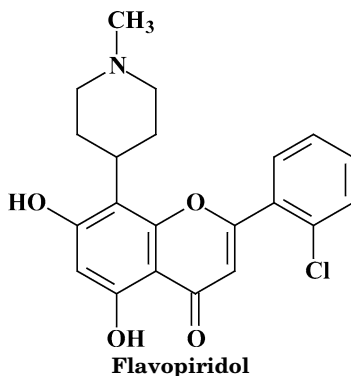
CGP64222

Scheme 43.

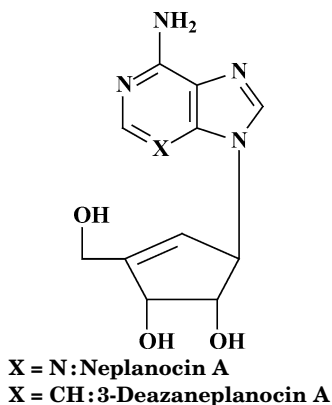
RKKRRQRRR of Tat, was also reported, on the one hand, to block the Tat/TAR interaction, and, on the other hand, to suppress HIV-1 replication.¹⁹⁵ We have demonstrated, however, that the peptoid CGP64222 owes its anti-HIV activity in cell culture primarily to an interaction with CXCR4, the coreceptor for X4 HIV strains,¹⁹⁶ which is, perhaps, not surprising given the structural similarity of CGP64222 to the other, polypeptidic, CXCR4 antagonists such as T22⁴⁰ and nona-arginine (ALX40-4C).⁴¹ In fact, Tat itself (following its extracellular release) has recently been shown to block CXCR4-dependent HIV-1 infection,¹⁹⁷ presumably through blockade of CXCR4 by the above mentioned 48–56 amino acid portion (RKKRRQRRR) of the molecule.

Flavopiridol (L86-8275, HMR1275) is a cyclin-dependent kinase (Cdk) and P-TEFb inhibitor, which is in clinical trials for the treatment of cancer because of its antiproliferative properties. P-TEFb is a protein kinase composed of Cdk9 and cyclin T1 and secures the elongation phase of transcription by RNA polymerase II (through phosphorylation of the carboxyl-terminal domain). Tat forms a triple complex with P-TEFb (composed of Cdk9 and cyclin T1) and the nascent transcript from the HIV-1 LTR promotor. Consistent with its ability to block P-TEFb, flavopiridol (**44**) was found to block Tat transactivation, and, concomitantly, also inhibited HIV replication.¹⁹⁸

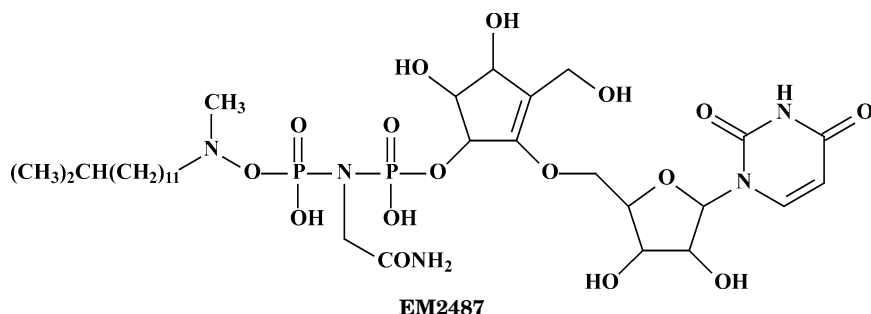
Capping and methylation of HIV pre-mRNAs are coupled to the elongation by polymerase II. Binding of the capping enzyme and cap methyltransferase to polymerase II depends on phosphorylation of its carboxyl-terminal domain, and capping and methylation reactions start as soon as the nascent pre-mRNA has attained a chain length of 19–22 nucleotides. It has been recently demonstrated that the cotran-



Scheme 44.



Scheme 45.



Scheme 46.

scriptional capping of HIV mRNA is stimulated by Tat, consequently to its binding to the capping enzyme.¹⁹⁹ These findings implicate capping as an elongation checkpoint critical to HIV gene expression, and thus corroborate earlier observations that S-adenosylmethionine-dependent methylations play an important role in the Tat-dependent transactivation of transcription from LTR.²⁰⁰ They also offer an explanation for the inhibitory effects of S-adenosylhomocysteine hydrolase inhibitors, such as neplanocin A and 3-deazaneplanocin A (**45**), on Tat-dependent transactivation and HIV replication.²⁰⁰

EM2487 (**46**), a natural product from *Streptomyces* was shown to inhibit HIV-1 replication through interference with the transcription process.²⁰¹ Whether this effect was, at least partially, mediated by inhibition of Tat could not be elucidated; in follow-up studies, EM2487 was also shown to inhibit HTLV-I (human T-lymphotropic virus type 1)

scissile substrate analogues for the HIV protease. All six licensed protease inhibitors (PIs) follow the same principle, that is they act as peptidomimetic inhibitors of HIV protease.²⁰⁵

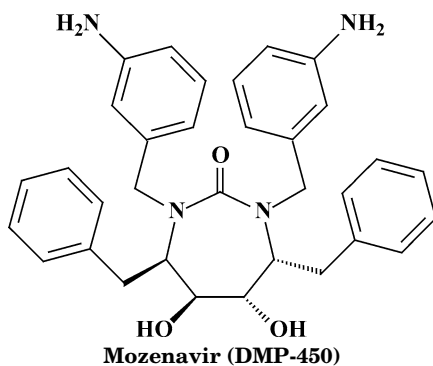
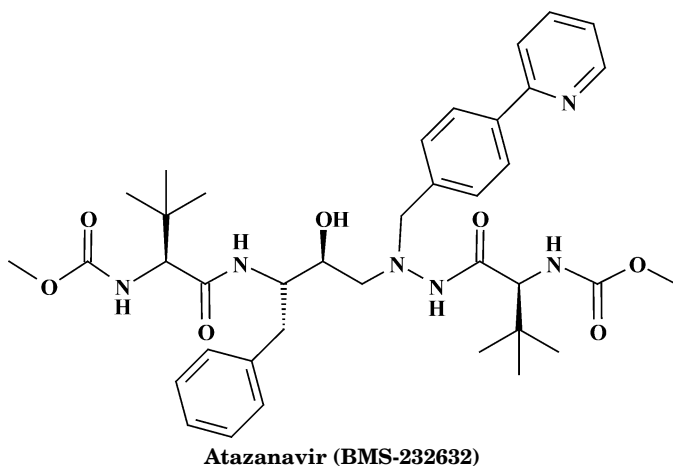
Lopinavir is co-dosed with ritonavir at 400/100 mg twice daily. The combination of ritonavir/lopinavir with stavudine and lamivudine has proven efficacious in the treatment of antiretroviral-naïve adults with HIV-1 infection.²⁰⁶ The reason for the co-dosing of lopinavir with ritonavir is that ritonavir strongly inhibits the metabolism of lopinavir and allows lopinavir to reach much higher plasma drug levels upon oral administration.²⁰⁷

In addition to the 2-hydroxyethylene scaffold, the L-mannaric scaffold has also proved to be a promising scaffold for the design of potent C2-symmetric HIV-1 protease inhibitors.²⁰⁸ These diol-based HIV-1 protease inhibitors show excellent antiviral activity in cell culture, also in the presence of 40% human serum.²⁰⁹ Their resistance profile, i.e., activity against HIV strains resistant to other peptidomimetic inhibitors of HIV protease remains to be determined.

Resistance mutations have been reported for most, if not all, peptidomimetic inhibitors of HIV protease. For instance, mutations L10F/I/RV, K20M/R, L24I, M46I/L, F53L, I54L/T/V, L63P, A71I/L/T/V, V82A/F/T, I84V and L90M, are associated with reduced susceptibility to lopinavir.²¹⁰ The emergence of resistance to the peptidomimetic PIs has prompted the search for new, non-peptidic inhibitors of HIV protease, that, in addition to a broader anti-HIV activity spectrum, might also offer increased oral bioavailability and/or pharmacokinetic properties. Examples of non-peptidic protease inhibitors of HIV protease include 4-hydroxycoumarins and 4-hydroxy-2-pyrones,²¹¹ sulfonamide-substituted derivatives,²¹² cyclic ureas (i.e., DMP-323 and DMP-450),^{213,214} cyclic cyanoguanidines,²¹⁵ aza-dipeptide analogues,²¹⁶ and tipranavir (PNU-140690), a sulfonamide-containing 5,6-dihydro-4-hydroxy-2-pyrone.²¹⁷⁻²¹⁹

The major advantage of the cyclic urea mozenavir (DMP-450) (**48**) is its substantial oral bioavailability observed in all species examined, including man.²¹⁴ DMP-450 has been the subject of phase I/II dose-escalating clinical studies and appears to have good antiviral activity and tolerability at all doses tested.

The new aza-dipeptide analogues combine excellent anti-HIV potency with high blood drug levels after oral administration, and, furthermore, they show no cross-resistance with saquinavir-resistant HIV strains.²²⁰ The prototype of the aza-dipeptide analogues (which contains an aza component attached to the hydroxyethylene scaffold) is atazanavir (BMS-232632) (**49**). Atazanavir is now in phase III clinical

*Scheme 48.**Scheme 49.*

trials; it has been accredited with a favorable resistance profile that does not parallel any of the other protease inhibitors currently in clinical use, as well as a favorable pharmacokinetic profile that would allow once-daily dosing.

Atazanavir, in combination with either stavudine, didanosine, lamivudine, zidovudine, nelfinavir, indinavir, ritonavir, saquinavir or amprenavir, yielded additive to moderately synergistic antiviral effects.²²⁰ Nelfinavir-, saquinavir-, and amprenavir-resistant HIV-1 strains remained sensitive to BMS-232632, while indinavir- and ritonavir-resistant viruses showed 6- to 9-fold changes in BMS-232632 sensitivity. On the other hand, BMS-232632-resistant (N88S, I84V) virus, se-

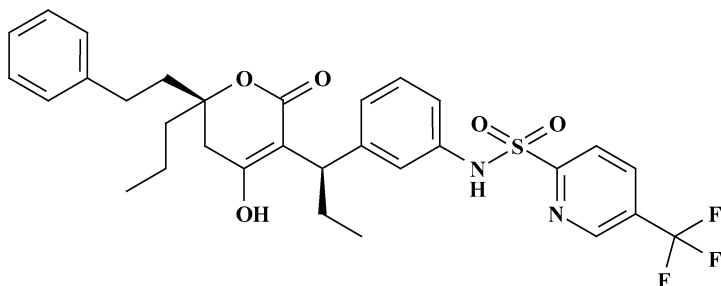
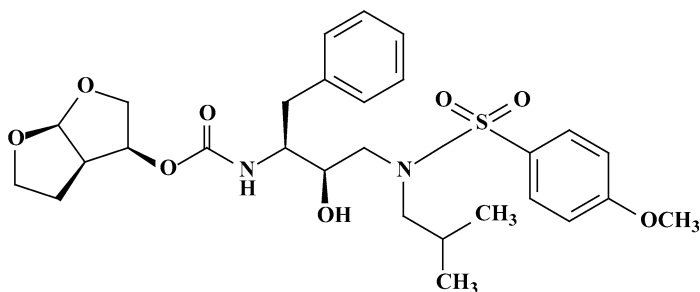
lected upon virus passage in the presence of the compound, remained sensitive to saquinavir, but showed various levels (0.1- to 71-fold decrease in sensitivity) of cross-resistance to nelfinavir, indinavir, ritonavir and amprenavir.²²¹

Atazanavir (BMS-232632) appeared to have a distinct resistance profile relative to the other protease inhibitors: analysis of the genotype profiles of 943 PI-susceptible and-resistant clinical isolates identified a strong correlation between the presence of amino acid changes at specific residues (10I/V/F, 20R/M/I, 24I, 33I/F/V, 36I/L/V, 46I/L, 48V, 54V/L, 63P, 71V/T/I, 73C/S/T/A, 82A/F/S/T, 84V, and 90M) and decreased susceptibility to atazanavir: while no single substitution or combination of substitutions was predictive of atazanavir resistance (> 3.0-fold reduction in susceptibility), the presence of at least five of these substitutions correlated strongly with loss of atazanavir susceptibility.²²²

Atazanavir once daily *versus* efavirenz once daily with fixed-dose zidovudine and lamivudine twice daily proved about equally efficacious in terms of virologic response (70% *versus* 64% with viral RNA < 400 copies/ml, respectively; 32% *versus* 37% with viral RNA < 50 copies/ml, respectively) after a 48-week treatment period.²²³ The signature mutation was I50L, but this mutation, while providing decreased susceptibility to atazanavir, brought about enhanced susceptibility to the other protease inhibitors.

Atazanavir offers several advantages over many of the currently available protease inhibitors.²²⁴ First, dosing is simple, two capsules once daily, which avoids a large pill burden. Second, dyslipidaemia (increased cholesterol and triglyceride levels) is not seen in both antiretroviral-naïve and experienced patients; and, third, although tentatively, insulin resistance and fat maldistribution may be less likely to occur.²²⁴

Also tipranavir (**50**)²¹⁷ showed low cross-resistance to HIV strains that were resistant to the established (peptidomimetic) inhibitors of HIV protease.^{218,219} Of 105 clinical HIV-1 samples with more than tenfold resistance to three or four of the peptidomimetic protease inhibitors and an average of 6 mutations per sample, 90% were susceptible to tipranavir, 8% had 4- to 10-fold resistance, and only 2% had more than 10-fold resistance, to tipranavir.²¹⁹ Typically HIV-1 isolates with the protease inhibitor resistance mutations L10I, K20M, M36I, L63P, A71V, V82T and L90M retained susceptibility to tipranavir.²²⁵ In addition, tipranavir retained marked activity against HIV-1 isolates derived from patients with multidrug resistance to other protease inhibitors,²²⁶ and might therefore be useful in combination

**Tipranavir (PNU-140690)***Scheme 50.***UIC-94003
TMC-126***Scheme 51.*

regimens with other antiretroviral agents for patients who already failed on other protease inhibitor-containing drug regimens. *In vitro*, tipranavir demonstrated synergistic activity with ritonavir against both ritonavir-sensitive and ritonavir-resistant HIV isolates.²²⁷

Recently, a novel HIV-1 protease inhibitor [UIC-94003 (TMC-126)], containing a *bis*-tetrahydrofuran-yl urethane and 4-methoxybenzenesulfonamide (**51**), and thus structurally related to amprenavir, was reported to be extremely potent against a wide spectrum of HIV-1 strains (EC_{50} : 0.3–0.5 nM), whether resistant to other PIs or not.²²⁸ Upon selection of HIV-1 in the presence of UIC-94003, mutants carrying a novel active-site mutation, A28S, in the presence of L10F, M46I, I50V, A71V and N88D, appeared. Modeling analysis revealed that close contact of UIC-94003 with the main chain of the protease active-site amino acid residues D29 and D30 differed from that of other PIs and may be important for its potency, particularly against drug-resistant HIV-1 variants.²²⁸

From computational studies of the free energy contribution and variability of the individual amino acid residues, it has been surmised that single drug-resistant mutations likely occur at not well conserved positions that are critical for drug binding but less important for substrate binding.²²⁹ Conversely, resistance-evading drugs should interact more strongly with the conserved residues. More specifically, improving interactions between the HIV protease inhibitors and residues L23, A28, G49, R87, and, particularly D29 in the HIV protease may possibly enhance their abilities to combat drug resistance.²²⁹

Independently of the HIV protease itself, proteasomes play a role in the processing of the gag polyprotein, and proteasome inhibitors, such as epoxomicin, have been shown to inhibit gag polyprotein processing as well as HIV maturation and release.²³⁰ While a potentially interesting approach, it remains to be seen whether inhibitors of the proteasome/ubiquitin system display sufficient specificity in their anti-HIV action so as to suppress virus replication without (overt) cytotoxicity.

XI. CONCLUSIONS

In recent years, an ever increasing number of compounds have been uncovered as anti-HIV agents targeted at virtually any step of the virus replicative cycle: adsorption, entry, fusion, uncoating, reverse transcription, integration, transcription (transactivation), and maturation. In addition to the “newer” NRTIs, NtRTIs, NNRTIs and PIs, various other compounds, i.e., those that are targeted at viral entry (i.e., CXCR4 and CCR5 antagonists) and virus-cell adsorption/fusion (i.e., compounds interacting with either gp120 or gp41), offer great potential for the treatment of HIV infections. Quite a number of compounds are capable of interacting with more than one target. Two examples in point are the dicaffeoyltartaric acid L-chicoric acid, and the nonapeptoid CGP 64222. L-chicoric acid was originally identified as an integrase inhibitor, and the nonapeptoid as a transactivation (Tat) antagonist, and their anti-HIV activity in acutely infected cells was ascribed to interference with the integration and transactivation process, respectively. However, L-chicoric acid primarily interacts as a virus adsorption inhibitor, and the nonapeptoid as a CXCR4 antagonist, and thus these compounds owe their anti-HIV activity mainly to interference with an early event (adsorption, entry) of the HIV replicative cycle.

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INHIBITORS OF HUMAN IMMUNODEFICIENCY VIRUS INTEGRATION

Daria J. Hazuda and Steven D. Young

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I. INTRODUCTION

The HIV-1 genome encodes three enzymes that are each essential for viral replication and thus considered attractive targets for chemotherapeutic intervention. The development of small, orally bioavailable drugs which inhibit two of these enzymes, the reverse transcriptase and protease, revolutionized the treatment of HIV-1 infection and AIDS.^{1,2} Antiretroviral regimens that include combinations of these agents are effective in reducing viral load and the morbidity and mortality associated with HIV-1 infection.³ However, given the persistent nature of the infection and the increasing appearance and spread of resistant variants as well as the toxicities associated with prolonged administration of many of the current treatment options, it is increasingly apparent that new chemotherapeutic strategies are necessary to manage chronic HIV-1 disease.⁴

HIV-1 integrase is the third of the virally encoded enzymes and therefore presents a potential opportunity for the development of novel antiretroviral agents. Integrase catalyzes the integration of the newly reverse transcribed HIV-1 DNA into the host cell's genomic DNA.⁵ Integration is a characteristic feature of retroviral replication required for stable maintenance of the viral genome as well as efficient viral gene expression. Integration is thus necessary for productive infection and integrase is the only protein known to be essential for catalyzing each of the required enzymatic events in the integration process.

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In addition to its role in integration, integrase may be important in reverse transcription and/or virus assembly, however, the catalytic activity of integrase is not required and the exact nature of these additional functions in the HIV-1 replication cycle has yet to be determined.

Integrase is expressed from the *pol* polyprotein and is cleaved by the HIV-1 protease to generate a 32 kDa, 288 amino acid residue protein. The three dimensional structure of the full length enzyme has not been elucidated, however, the enzyme is composed of three independent domains whose structures have each been determined.⁶ The N-terminal domain (amino acid residues 1–50) contains a highly conserved “HH–CC” motif or zinc binding domain. Residues 213 to 288 form the C-terminal DNA interaction domain which participates in binding to the viral DNA. Amino acids 51 through 212 constitute the catalytic core of the enzyme, encompassing the critical aspartate (D64 and D116) and glutamate (E152) residues that are conserved among all retroviral integrases and many phosphotransferases. As with many phosphotransferases, the three acidic residues mediate the coordination of the divalent metal ion cofactor(s) required for catalytic function and are thus essential for phosphodiester bond cleavage/formation.⁷ Mg^{+2} is generally believed to be the biologically relevant metal ion co-factor, however, Mn^{+2} is often used in *in vitro* assays of integrase function to increase overall enzymatic activity.⁸

In the context of HIV-1 replication, integration occurs as a staged, multistep process (Fig. 1). The first and most fundamental step in this process is the formation of a stable complex with specific sequences at the long terminal repeat (LTR) regions of the viral DNA.⁹ It is only in the context of a complex with these DNA sequences that the enzyme performs the two specific catalytic steps required for integration.

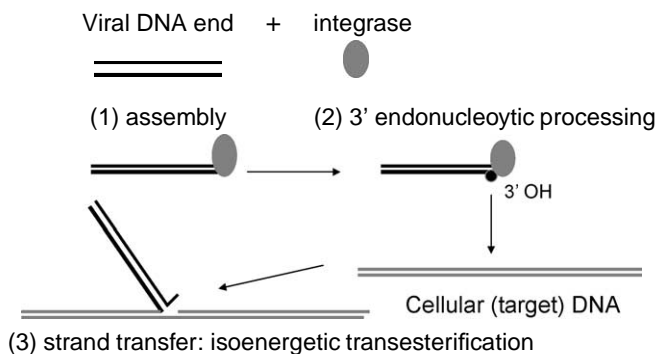


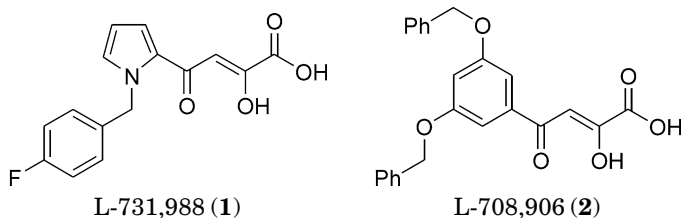
Figure 1.

In the first catalytic reaction, endonucleolytic processing, integrase specifically cleaves the terminal 3' GT dinucleotide, leaving a recessed 3'-OH at both 3' termini. The integrase/DNA preintegration complex or "PIC" translocates to the nucleus. Integrase then performs the second catalytic function, strand transfer, whereby the host cell or target DNA is nicked and the 5' end is covalently linked to the processed 3' end of the viral DNA. This latter reaction proceeds by an isoenergetic transesterification process in which the energy of phosphodiester bond breakage is conserved for strand joining.

Although many compounds have been identified as inhibitors of integrase in biochemical assays, the antiviral activity of most of these inhibitors has been shown to be independent of integrase¹⁰ and thus only a very distinct subset of these compounds have been shown to inhibit HIV-1 replication by preventing integration. Several general reviews have been published on inhibitors of HIV-1 integrase providing a thorough historical perspective on the field.¹¹ This review will therefore attempt to highlight some of the more recent advances with a focus on those small molecules that have been validated as *bona fide* integration inhibitors and their potential as drug development leads.

II. 4-ARYL-2,4-DIKETOBUTANOIC ACIDS: BIOCHEMICAL AND ANTIVIRAL PROPERTIES

The 4-aryl-2,4-diketobutanoic acids (or diketo acids) L-731,988 (**1**) and L-708,906 (**2**) (Fig. 2) were the first integrase inhibitors demonstrated to have activity against HIV-1 replication in cell culture directly as a consequence of their effect on integration.¹² The validation of integrase as the antiviral target of these inhibitors was established in two ways:



IC ₅₀ = 0.01 μ M	Enzyme inhibition (strand transfer)	IC ₅₀ = 0.04 μ M
IC ₉₅ = 9.6 μ M	Antiviral activity (iiiib virus/MT4 cells)	IC ₉₅ = 17.4 μ M

Figure 2.

- (1) by identifying key mutations in integrase and demonstrating an association with the resistant phenotype and
- (2) by correlating the unique inhibitory mechanism observed in biochemical assays with the effects observed in HIV-1 infected cells.¹¹

The 4-aryl-2,4-diketobutanoic acids (1) and (2) were shown to be mechanistically distinct from previously identified integrase inhibitors which affect integrase function by preventing the interaction (or assembly) with DNA.¹³ Both in biochemical assays and in HIV-1 infected cells (1) and (2) selectively inhibit the strand transfer activity of integrase, exhibiting a greater than fifty-fold difference in assays measuring strand transfer versus 3' end processing, respectively.^{12,14}

The elucidation of this distinct mechanism of action of the 4-aryl-2,4-diketobutanoic acids as specific inhibitors of the strand transfer activity of integrase and the demonstration that this mechanism is effective in blocking HIV-1 replication was critical to validating this approach to exploiting integrase as a chemotherapeutic target. These compounds and structurally diverse strand transfer inhibitors that will be described below stall the integration process in infected cells and allow the unintegrated viral DNA in the nucleus to be efficiently metabolized by cellular recombination and repair enzymes.^{12,15} Cellular processes which include non-homologous end-joining, convert the linear integration competent viral DNA into a variety of circular products resulting in an effective and irreversible block of viral replication.¹⁶ The circular products that are observed in HIV-1 infected cells treated with integrase inhibitors reproduce the phenotype that has been described for HIV-1 integrase mutants defective in integration.¹⁷

III. 4-ARYL-2,4-DIKETOBUTANOIC ACIDS: MOLECULAR MECHANISM

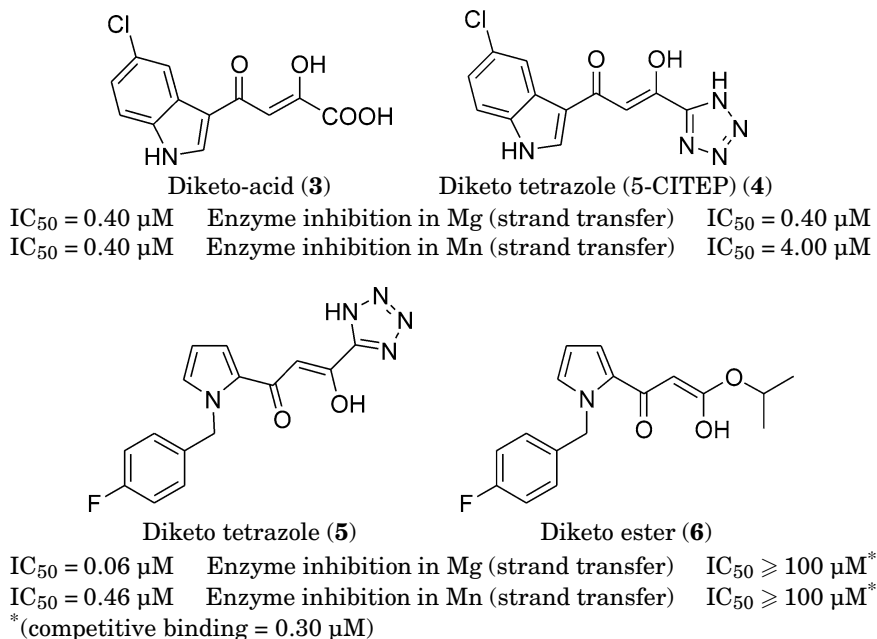
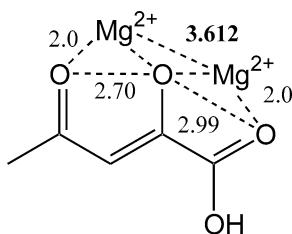
The molecular basis that underlies the activity of the 4-aryl-2,4-diketobutanoic acids as strand transfer selective inhibitors has recently been explored and these studies have uncovered several unique characteristics that distinguish these compounds and analogs that share this distinct mechanism.^{14,18}

First, high affinity binding to integrase requires assembly of a strand transfer competent complex on the viral DNA. There is approximately a three-order of magnitude difference between the affinity of the 4-aryl-2,4-diketobutanoic acids for the isolated, free enzyme and integrase in

the context of the assembled complex. The tight binding interaction between these inhibitors and the complex is highly restricted to integrase associated with the HIV-1 specific LTR sequence. Once bound to the complex, the inhibitors compete for binding to the target DNA substrate thus leading to the observed effect on strand transfer. The evidence that strand transfer inhibitors require binding to the viral donor substrate and are competitive with respect to target substrate is consistent with the observation that such compounds are ineffective in disintegration assays. Disintegration substrates are designed to represent integration intermediates in which the donor and target DNA's are covalently linked thus simultaneously occupying both substrate binding sites on the enzyme.¹⁹ These observations suggest integrase may adopt a unique conformation when associated with the viral DNA substrate and demonstrate that assays that evaluate different stages in the integration process can be sensitive to distinct types of inhibitors. The results may also in part explain why many biochemical assays designed to assess either 3' end processing or disintegration have failed to identify inhibitors of strand transfer.

Second, a variety of experimental evidence suggests there is a direct interaction between the essential diketo carboxylate (or isosteric replacement) motifs of these inhibitors and divalent metal ion(s) in the integrase active site.¹⁸ Binding of the 4-aryl-2,4-diketobutanoic acids to the integrase/DNA complex is completely dependent on divalent metal and the activity of structurally related analogs with acid replacements can exhibit specific metal dependence. For example, a direct interaction between the inhibitor carboxylate or isosteric heterocycle with a metal would account for the distinct metal preferences observed with compounds such as **(1)** and **(3)** versus **(4)** and **(5)** (Fig. 3). Although some non-acidic replacements like the ester **(6)** shown in Fig. 3 exhibit only a modest loss in affinity for the integrase complex, remarkably these compounds are ineffectual when evaluated in strand transfer assays under identical conditions.¹⁷ These results suggest that the interaction with and sequestration of the catalytic metal by the diketo carboxylate moiety underlies the inhibitory affect in these compounds. This mechanism of action is consistent with the evidence that mutations engendering resistance to these inhibitors are located proximal to the active site residues that coordinate the active site metal cofactors in integrase.¹²

Based on these observations and data from X-ray crystallography studies of integrase and other phosphotransferase enzymes a model for the interaction of these compounds and the active site metals has been proposed (Fig. 4,¹⁸). This model is consistent with the require-

**Figure 3.****Figure 4.** Model for interaction with divalent metals.

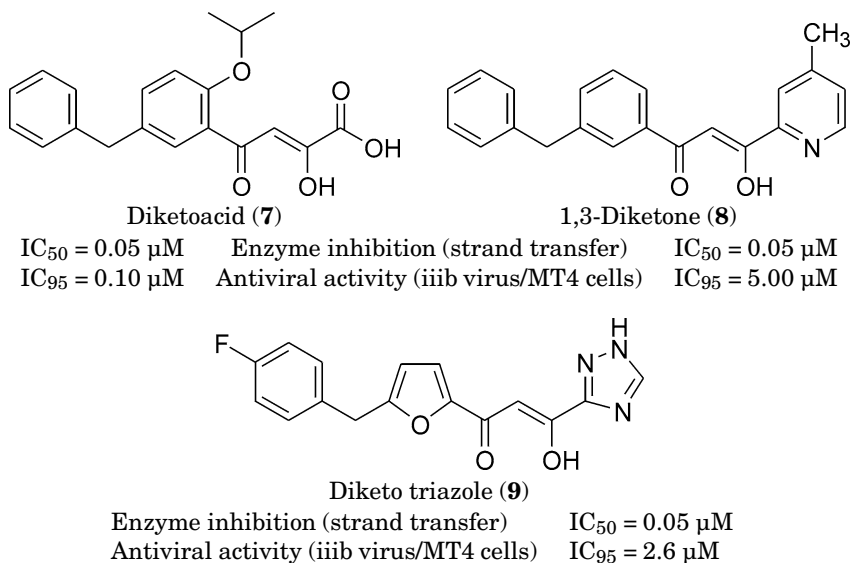
ment for the acid functionality and observed metal dependence of these inhibitors. In this model, the inhibitor coordinates two metals bound by the conserved active site residues, D64, D116 and E152. The bond lengths and angles for the acid moiety used in developing this model are based on the crystal structure of 5-CITEP (4).²⁰ As in the 5-CITEP structure, the diketo carboxylate portion of the inhibitor is planar. This assumption is consistent with the structure activity analyses for these

compounds that will be discussed below. Assuming 2.0 Å as the bond length between oxygen atoms of the inhibitor and metals and that each metal is equidistant from either the ketone or carboxylate oxygen atoms, the distance between the metals is calculated to be 3.61 Å. This calculation is thus in agreement with the distance of 3.62 Å observed between the two active site metals in the X-ray structure of ASV integrase.²¹

It is worth noting that, consistent with the proposed mechanism of action of the 4-aryl-2,4-diketobutanoic acids, similar compounds have been identified which also inhibit other metal dependent phosphotransferase enzymes including the RNase H activity of HIV-1 reverse transcriptase.²² Although HIV-1 RNase H and integrase exhibit minimal sequence homology, the proteins belong to the same general family of phosphotransferases and share a similar active site architecture including the conserved metal binding motif. The 4-aryl-2,4-diketobutanoic acids that inhibit RNase H also exhibit metal dependent activity as observed for HIV-1 integrase. These findings suggest the general mechanism exemplified by these compounds can be exploited to identify inhibitors of a variety of clinically interesting drug discovery targets.

IV. NOVEL 4-ARYL-2,4-DIKETOBUTANOIC ACIDS AND 1,3-DIARYL-1,3-PROPANEDIONE

The 4-aryl-2,4-diketobutanoic acids L-731,988 (**1**) and L-708,906 (**2**) were identified through sample collection screening using a biochemical assay specifically designed to identify inhibitors of the strand transfer activity of integrase.¹² Although (**1**) and (**2**) exhibit potent activity in this biochemical assay, the antiviral activity of these compounds in cell culture assays of HIV-1 replication is modest (IC₉₅'s of 10 and 17 µM, respectively). Modifications to these inhibitors have been shown to have a dramatic affect on potency and have yielded new 4-aryl-2,4-diketobutanoic acids with greatly improved antiviral activity.²³ Several integrase inhibitors in the 2,4-diketobutanoic acid class have been described which exhibit antiviral activities *in vitro* that are comparable to many clinically effective antiretroviral agents. For example, optimization of the 4-aryl portion in this series led to compound (**7**) which is reported as having an IC₅₀ of 50 nM for strand transfer inhibition and antiviral activity in the HIV-1 replication assay with an IC₉₅ of 0.10 µM (Fig. 5). By comparison, the HIV-1 protease inhibitor indinavir has an IC₉₅ = 0.055 µM in the same assay (HIV_{iiiB} virus in MT4 cells).

**Figure 5.**

In this series the carboxylate can be replaced with an electron pair donor heterocycle as demonstrated by compound (8) (Fig. 5).²⁴ Replacement of the carboxylate with a properly oriented Lewis basic heterocycle such as the pyridine of (8) is also exemplified by the 1,2,4-triazole compound (9) (Fig. 5). Shionogi and Merck have disclosed a variety of 4-aryl-2,4-diketobutanoic acids and structurally related 1,3-diaryl-1,3-propanedione inhibitors of HIV-1 integrase.^{24,25} The 4-(5-chloroindol-3-yl)-2,4-dioxobutanoic acid (3) is an inhibitor of HIV integrase strand transfer activity with an inhibitory $IC_{50} \sim 1 \mu M$. The Brønsted acid equivalent tetrazole analog of (3), compound (4) or 5-CITEP, 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2H-tetrazol-5-yl)propanedione also inhibits of strand transfer and the tetrazole functionality confers a strict dependence on Mn^{+2} to the activity.¹⁸ Compound (4) is poorly active when assayed in the presence of Mg^{+2} and is completely devoid of antiviral activity. Although the related diketone triazole, 3-(2-(5-(4-fluorobenzyl)furyl))-1-(3-triazolyl)propanedione (9), also exhibits a preference for Mn^{+2} , perhaps due to increased intrinsic activity, the compound inhibits strand transfer when assayed in Mg^{+2} and exhibits antiviral activity in vitro ($IC_{95} = 2.6 \mu M$).²⁶

5-CITEP, compound (4) is the only active site inhibitor of integrase for which structural information is available to date.²⁰ In the X-ray co-crystal structure with the catalytic core domain of integrase, (4) is

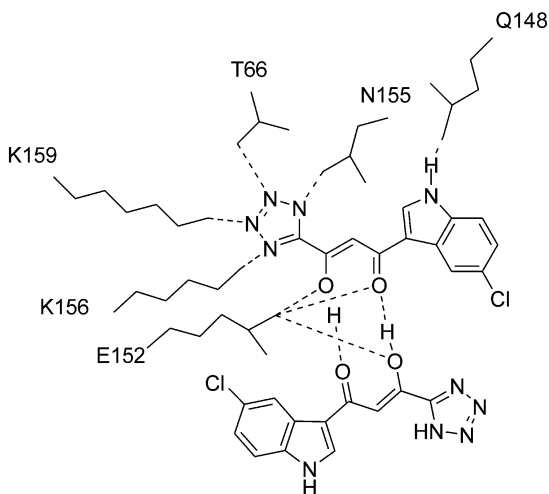


Figure 6.

arranged within the active site between the catalytic residues D64, D116 and E152. Hydrogen bonds are formed between a variety of active site residues and the indole nitrogen, all four tetrazole nitrogens and the dicarbonyl oxygens. Interestingly, (4) makes certain contacts with residues that are identical or close to those predicted to be important for the 4-aryl-2,4-diketobutanoic acids on the basis of mutations shown to engender resistance (e.g., N155 and T66, Fig. 6). While the co-crystal structure is consistent with some of the biochemical properties of these inhibitors, no metal ions are engaged by either the dicarbonyl oxygens or tetrazole nitrogens in the molecule and there is no DNA in the structure, therefore important features relevant to the function of this class of integrase inhibitors are not fully represented. Together with the biochemical data, the structural information indicates assembly of an appropriate complex with DNA may be required to

- (1) stabilize divalent metal binding in the integrase active site and/or
- (2) recapitulate the relevant binding mode of these inhibitors.

V. STRUCTURALLY DIVERSE INHIBITORS: 1,6-NAPHTHYRIDINES

Although a variety of potent diketone type inhibitors such as those described above have shown promising activity against HIV-1 in stan-

dard cell culture assays, these compounds tend to be highly protein bound and exhibit significantly reduced activity when assayed in the presence of human serum.²⁶ For example, compound (**9**) which has sub-micromolar antiviral activity when assayed in 10% fetal bovine serum, has an IC₉₅ of 12 μ M when assayed in the presence of human serum (Hazuda, unpublished observations). Therefore, structurally diverse analogs with isosteric replacements for the entire 2,4-diketobutanoic acid pharmacophore have been designed (Fig. 7).²⁷ Among these include novel naphthyridine analogs which retain potency both in enzymatic activity and antiviral assays and exhibit improved protein binding and pharmacokinetic properties relative to previously described inhibitors. In particular, one such analog has recently been shown to be efficacious in SHIV infected rhesus monkeys.

Starting with the 4-aryl-2,4-diketobutanoic acid (**1**), a novel set of bioisosteric diketo acid equivalents were designed and prepared based on structure activity analyses which suggested a number of assumptions regarding the active conformation of the pharmacophore.²⁷ The 1,3-diketoacid moiety enolizes at the α -position, and the resultant conjugated *Z*-4-oxo-2-hydroxy-2-butenic acid side chain is coplanar with the central benzene ring. Stepwise replacement of the hydroxyl groups

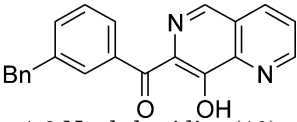
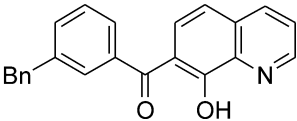
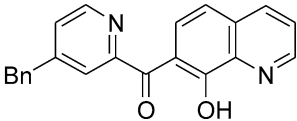
Compound	Strand transfer inhibition IC ₅₀ (μ M)	Antiviral activity IC ₉₅ (μ M)	Cytotoxicity (μ M)
 1,6-Naphthyridine (10)	0.04 \pm 0.02 (<i>n</i> = 4)	6.20 \pm 1.8 (<i>n</i> = 3)	12.50
 Quinoline (11)	0.37 \pm 0.06 (<i>n</i> = 2)	5.00 (<i>n</i> = 1)	1.25
 Quinoline (12)	0.05 \pm 0.01 (<i>n</i> = 2)	2.50 (<i>n</i> = 1)	2.50

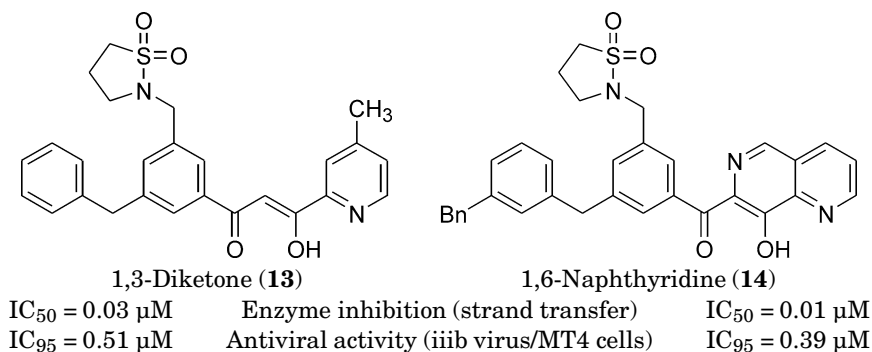
Figure 7.

with more pharmacologically suitable functionalities led to the identification of the series of heteroaromatic inhibitors shown in Fig. 7. In these compounds the aromatic nitrogen acts as the Lewis base equivalent of the corresponding carboxylate oxygen in the diketobutanoic acids such as (**1**).

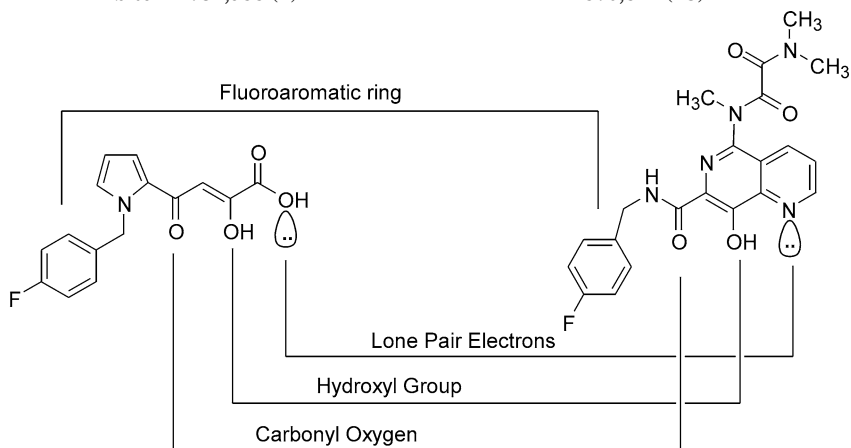
As summarized in Fig. 7, the 1,6-naphthyridine (**9**) exhibits good potency in the strand transfer activity assay (IC_{50} 40 nM) and antiviral activity comparable to the prototypical 4-aryl-2,4-diketobutanoic acid inhibitor L-731,988 (**1**). The quinoline (**10**) is 9 fold less potent than the corresponding naphthyridine analog, however shifting the aromatic nitrogen to the central benzene ring restores potency in the strand transfer assay, e.g., quinoline (**11**) is comparable to naphthyridine (**9**). The incorporation of a heteroatom would allow for adoption of coplanar conformations between the central benzene ring and the heteroarylpyridine and avoid the unfavorable conformational bias presented by the carbon counterpart in (**10**). These results are therefore in accordance with the assumption that a coplanar arrangement of the central phenyl ring (or pyridine ring) and the naphthyridine ring (or quinoline ring) is important for activity. In addition, the 8-hydroxy-[1,6]naphthyridine (**9**) is significantly less cytotoxic than the corresponding 8-hydroxyquinolines (**10**) and (**11**) (12.50 μ M vs. 1.25 and 2.50 μ M, respectively). This may be related to the observation that unsubstituted 8-hydroxy-[1,6]naphthyridines have much reduced affinity for metal ions relative to the unsubstituted 8-hydroxyquinolines.

It is interesting that in part the structure activity relationship observed in either the 4-aryl-2,4-diketobutanoic acid or diketone class may be translated to the 1,6-naphthyridine series; e.g., the diketone (**12**) and the naphthyridine (**13**) shown in Fig. 8 each incorporate a sultam substitution at the 3 position of the central phenyl ring. Naphthyridine (**13**) exhibits good activity in both the strand transfer and antiviral activity assay, with an IC_{50} of 10 nM in the strand transfer and an IC_{95} of 390 nM in cell culture without toxicity ($IC_{50} > 12.5 \mu$ M).

Compound (**13**) and related 1,6-naphthyridines such as (**14**) (Fig. 9) exhibit reduced potency against viruses engineered to contain integrase mutations such as T66I/S153Y and T66I/M154I which were previously shown to confer resistance to the 4-aryl-2,4-diketobutanoic acid inhibitors. These results demonstrate that the antiviral activity of the 1,6-naphthyridines is due to their effect on integrase and suggest the naphthyridines bind to and inhibit HIV-1 integrase in a fashion indistinct from that of the 4-aryl-2,4-diketobutanoic acids. These observations are also consistent with a model in which the 8-hydroxy-[1,6]naphthyridine group as in (**14**) is the structural and func-

**Figure 8.**

2,4-Diketobutanoic acid strand transfer inhibitor L-731,988 (**1**) 8-Hydroxy-1,6-naphthyridine-7-carboxamide L-870,812 (**15**)

**Figure 9.**

tional equivalent of the diketo carboxylate motif in the original strand transfer inhibitors such as L-731,988 (**1**) (Fig. 9). Like the 4-aryl-2,4-diketobutanoic acids, the 1,6-naphthyridines are selective inhibitors of strand transfer which bind with high affinity to the integrase complex in a divalent metal dependent manner.

The 8-hydroxy-[1,6]naphthyridines (**13**) and (**14**) have potent antiviral activity and improved pharmacokinetic properties relative to the 4-aryl-2,4-diketobutanoic acids and diketones.²⁶ For example, when

(**13**) was administered intravenously to rats at 2 mg/kg, it exhibited a half-life ($T_{1/2}$) of 9.7 hours with a moderate clearance rate (Cl_p) of 2.98 mL/(min kg). In rats, a 10 mg/kg dose of (**13**) administered orally, achieved a peak plasma level (C_{max}) of 1.17 μ M at 60 minutes and maintained a plasma concentration greater than 0.8 μ M throughout the first 6 hours. This data suggests integrase strand transfer inhibitors such as these should prove effective in reducing viral replication HIV-1 infected patients. Recently compound (**14**) has been shown to mediate the sustained suppression of viral replication and the preservation of CD4 cell levels in an SHIV immunodeficiency infection model of rhesus macaques thus providing the first demonstration of *in vivo* efficacy for this novel class of antiretroviral agents.²⁶

VI. CONCLUSIONS

Of the three HIV-1 enzymes that are potential targets for therapeutic intervention, the identification of small molecule inhibitors of integrase has proven the most elusive. Early lead compounds affected integrase activity *in vitro* but did not have activity in cell culture or the observed antiviral activity was a consequence of mechanisms unrelated to integration. Recent understanding of the mechanism of action intrinsic to different classes of integrase inhibitors, i.e., inhibition of assembly, 3' end processing and strand transfer, has facilitated the identification of compounds that are *bona fide* inhibitors of integration in cell culture and proven that inhibiting integrase strand transfer leads to a profound antiviral effect. Studies on these inhibitors have led to additional insights into the mechanism of integrase activity both *in vitro* and in HIV-1 infected cells. Most importantly, the demonstration that strand transfer inhibitors with potent activity against HIV-1 in cell culture and favorable pharmacokinetic properties are efficacious in a rhesus model suggests this mechanism will yield novel antiretroviral agents and provide a new class of therapies to treat HIV-1 infection.

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NON-PEPTIDIC PROTEASE INHIBITORS (NPPIs): TIPRANAVIR

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I. BACKGROUND

Tipranavir (tipranavir, formerly PNU-140690) is a potent, highly selective, orally bioavailable, non-peptidic protease inhibitor (NPPI) that was discovered using a high throughput screening process. Structurally, tipranavir belongs to a new class of sulphonamide-containing dihydropyrones related to coumarin which inhibit HIV protease, an aspartyl protease essential for viral maturation (Figure 1).¹

Mutations in the HIV protease gene lead to the emergence of viral strains which are resistant to protease inhibitors.² Initially it was thought that the protease gene would be less liable to mutate than the HIV reverse transcriptase gene making resistance to PIs less likely to develop; clinical experience has proved otherwise. Resistance to PIs develops as a result of the evolution of polymorphisms and amino acid substitutions within the protease enzyme due to viral replication in the presence of sub-optimal drug selection pressure.

During the mid 1990s, PI-based regimens were shown to be effective in suppressing viral replication to levels below the detection limits of the assays available at the time.^{1,3} This approach to HIV therapy has resulted in a decline in mortality and morbidity over recent years.^{1,4,5} Unfortunately in a significant proportion of patients who have failed their first and subsequent PI regimens, their viruses develop broad protease inhibitor resistance, and hence require new treatment options.⁵

Tipranavir is a new, non-peptidic PI that should become a valuable addition to the anti-HIV armamentarium, particularly for patients who have experienced treatment failure with currently available PIs. Preliminary clinical data on the use of tipranavir in treatment naive patients suggest that it may also be useful in this patient population.

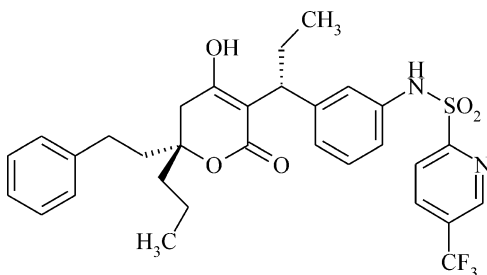


Figure 1. Chemical structure of tipranavir: single stereoisomer 3R, 6R, TPV (free acid form). Chemical name: [R-(R*,R*)]-N-[3-[1-[5,6-Dihydro-4-hydroxy-2-oxo-6-(2-phenylethyl)-6-propyl-2H-pyran-3-yl]propyl]phenyl]-5-(trifluoromethyl)-2-pyridinesulfonamide.

II. FORMULATIONS

Three oral formulations of tipranavir have been developed: a disodium salt; a hard filled capsule (HFC); and a soft gel capsule, which contains tipranavir free acid in a self-emulsifying drug delivery system (SED DS). The bioavailability of tipranavir administered as SED DS was two-fold higher than that obtained when HFCs were used and showed improved tolerability in patients. The 250 mg SED DS capsule is the tipranavir formulation which will be used in all Phase III clinical trials.

III. PHARMACODYNAMICS

Tipranavir has a high affinity for HIV-1 and HIV-2 proteases: the K_i is 8.9 pM and < 1 nM, respectively.^{6,7} Tipranavir is highly selective for the HIV protease enzyme since human aspartyl proteases, cathepsins D or E and pepsins are only inhibited in the μ M range.¹

A. Inhibitory/Effective Concentrations

Tipranavir exhibited antiviral activity in HIV_{IIIB}-infected H9 cells: the 50% effective concentration (EC_{50}) was 0.03 μ M and the 90% effective concentration (EC_{90}) was 0.1 μ M.⁶⁻⁸ The replication of both laboratory and clinical viral strains in primary peripheral blood mononuclear cells (PBMCs) was suppressed by tipranavir. Infection of PBMCs with laboratory strains, HIV_{IIIB}, 14aPre and N70, was inhibited by tipranavir. The mean EC_{90} was 0.18 μ M. The mean EC_{90} for multi-drug resistant clinical isolates ranged from 0.31 to 0.86 μ M.⁹

B. Effect of Plasma Proteins

Tipranavir is extensively bound to plasma proteins (> 99.9%) in a concentration independent manner. However, its activity is not significantly affected by the presence of high concentrations of human plasma proteins.¹⁰ The EC_{90} of tipranavir against HIV-1_{IIIB} in H9 cells and HIV-1_{JR-CSF} in peripheral blood mononuclear cells was increased between 1.7- and 6.2-fold by the presence of human plasma, human plasma albumin, or α 1-acid glycoprotein in the culture medium. This is consistent with the 3- to 4-fold shift in EC_{50} when 75% human plasma is added to *in vitro* drug susceptibility assays. The protein-adjusted EC_{90} for tipranavir (correction factor 3.75) was between 0.5 and 2.0 μ M for the majority of HIV clinical isolates.

C. Combination of Ritonavir with Tipranavir *in vitro*

The effects of tipranavir and ritonavir on ritonavir-sensitive and ritonavir-resistant clinical isolates have been investigated *in vitro*.⁸ The concentrations of the PIs used were less than the plasma levels that can be achieved in patients taking therapeutically effective doses. Over the time course of an acute infection of PBMCs with a ritonavir-sensitive HIV-1 isolate, the combination of tipranavir and ritonavir was significantly more effective than exposure to either PI alone ($p < 0.01$). Tipranavir plus ritonavir was also significantly more active against the ritonavir-resistant isolate than each PI alone. An unexpected finding was that the greatest synergistic effects were observed in the cultures infected with a ritonavir-resistant isolate. No significant antagonism was observed.

IV. PHARMACOKINETIC PROFILE

A. Pharmacokinetics in Healthy Volunteers

Tipranavir is orally bioavailable in both animals and man.⁷ Single dose (range: 100 to 2000 mg) studies of tipranavir in healthy volunteers have been conducted.^{7,11} When single doses ≥ 500 mg were administered, the drug plasma concentrations after 8 h were in excess of 1 μM . Single dose pharmacokinetics were linear, but there was a considerable degree of inter-individual variation in terms of the pharmacokinetic parameters.¹¹ Peak plasma concentrations occurred within 1 to 5 h after capsule intake. Oral clearance after a single dose was 18 L/h.

Plasma drug concentrations in healthy volunteers who took multiple doses of tipranavir (300, 600, 900, 1200, 1600, or 2000 mg for nine days) were lower than would be predicted from the single dose data, presumably due to hepatic enzyme induction. Steady state was reached within six days of initiating the administration; the half-life ($T_{1/2}$) of tipranavir at steady state was approximately 4 h. Trough plasma concentrations (C_{\min}) averaging approximately 1 μM were achieved at doses ≥ 900 mg tipranavir TID. The pharmacokinetics of tipranavir without ritonavir were similar in healthy volunteers and in HIV positive individuals when they received similar doses of tipranavir.^{1,12}

B. Metabolism/Effect of Food

Tipranavir is a substrate for, and inducer of, cytochrome P450 3A (CYP3A4) in animals and in man.¹³ Glucuronidation of tipranavir appears to be the main clearance mechanism. Preliminary assessments

indicate no clinically significant effect of food with tipranavir although there is a slight increase in absorption with the SEDDS formulation following a high fat meal.

C. Co-Administration with Ritonavir

Co-administration of low dose ritonavir with all of the currently licensed PIs except nelfinavir increases the plasma AUCs of the individual PIs by several fold ('PI boosting'); the co-formulation of lopinavir boosted by ritonavir has been approved by several regulatory agencies world-wide.^{14,15} The AUC of tipranavir is increased between six- and nine-fold in the presence of ritonavir (Figure 2).

The pharmacokinetics of tipranavir (SEDDS) and ritonavir in healthy volunteers (randomized = 113; completed study = 95) were studied in trial BI 1182.5, which was an open-label, parallel group, multiple-dose study.¹⁶ On study days 1–11, tipranavir (250, 500, 750, 1000, or 1250 mg BID) was taken. On study days 12–32, ritonavir was added to the regimen (100 or 200 mg BID). Intensive pharmacokinetic studies were conducted on study days 11, 18, 25, and 32. Apart from the tipranavir 250 mg/ritonavir 200 mg dose, all of the tipranavir/ritonavir

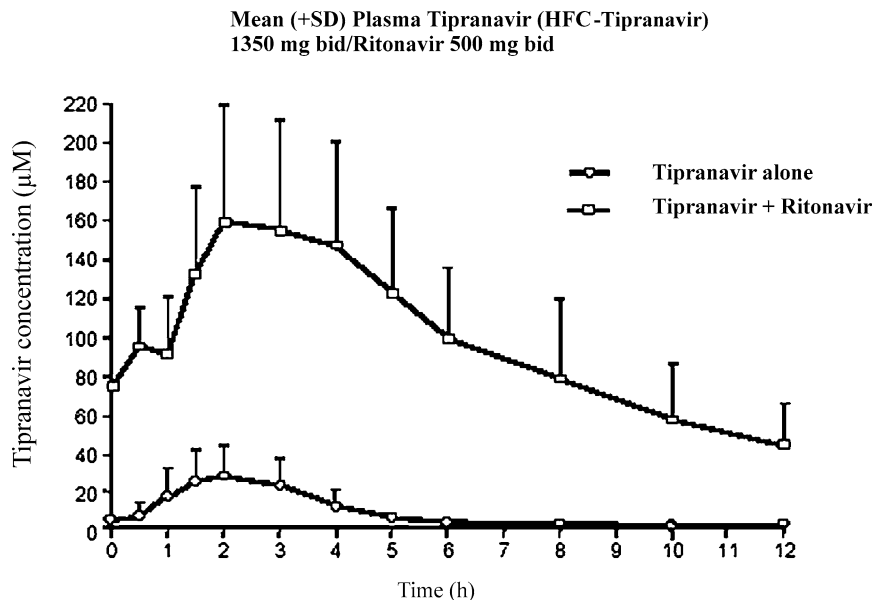


Figure 2. Effect of ritonavir on tipranavir exposure.

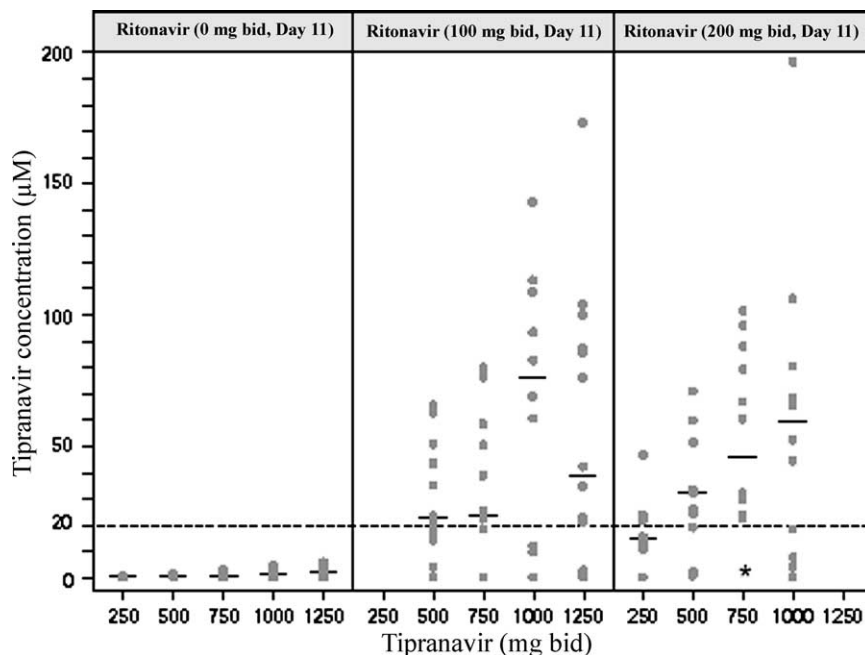


Figure 3. Tipranavir morning trough concentrations in the presence and absence of ritonavir: (---) 20 µM target, (■) data point for individual subjects, (*) subject that experienced emesis within 2 h of treatment administration, and (—) median.

doses generated plasma trough levels (C_{\min}) which were above the target level of 2.0 µM, which is 10-fold higher than the protein adjusted EC_{90} for multiple PI-resistant HIV. The tipranavir C_{\max} increased approximately four-fold in the presence of co-administered ritonavir (Figure 3).

The effects of the two PIs on CYP3A activity were also investigated in trial BI 1182.5, using the erythromycin breath test (ERMBT). The results of the ERMBTs in healthy volunteers confirmed that tipranavir is an inducer of CYP3A4 and ritonavir is a potent inhibitor of this enzyme system but the combined effect is inhibition. This mixed induction and inhibition of the CYP3A4 enzyme system results in lower plasma concentrations of ritonavir when administered with tipranavir: 100 mg ritonavir, when co-administered with 500 mg of tipranavir, has similar plasma concentrations as 60 mg ritonavir administered without tipranavir. Likewise, 200 mg ritonavir co-administered with tipranavir yields similar plasma drug concentrations as to 120 mg ritonavir without tipranavir. Based on these data, all further clinical

studies of tipranavir have used the combination of tipranavir with low-dose ritonavir.

D. Co-Administration of Tipranavir and Ritonavir with other Antiretroviral Agents

Large pharmacokinetic changes in tipranavir levels are observed if 100 mg ritonavir is co-administered with a second agent than with 200 mg ritonavir. If 100 mg ritonavir is co-administered, induction of the CYP3A4 pathway can still occur, if 200 mg ritonavir is co-administered, the CYP3A4 pathway is completely blocked and no induction occurs. Interaction studies in patients receiving stable background NRTIs and NNRTIs (trial BI 1182.6) have been performed, but were not designed to test for the effect of these ARVs on the steady-state pharmacokinetics of tipranavir.¹⁷ A summary of the interaction of tipranavir/ritonavir with other antiretrovirals is shown in Table 1.

E. Co-Administration of Tipranavir and Ritonavir with Nucleosides

Tipranavir/ritonavir co-administration does not result in clinically significant changes in pharmacokinetic parameters for lamivudine, stavudine, or didanosine. Although reductions in abacavir and zidovudine levels were seen with these agents when they were co-administered with tipranavir/ritonavir, this was not thought to be of clinical significance—in trial BI 1182.6 and subsequent studies there was no adverse effect on viral load seen with these drug combination.¹⁷ There was poor correlation between pharmacokinetic parameters of nucleoside agents and their active intracellular dideoxynucleoside triphosphate concentrations. Because of potential for dissolution interaction between didanosine, it is recommended that tipranavir/ritonavir combination is administered 4 hours apart from didanosine. When tipranavir is administered with 200 mg ritonavir no clinically significant changes in tipranavir concentrations were observed in drug interaction studies with didanosine, tenofovir, or zidovudine.

F. Co-Administration of Tipranavir and Ritonavir with Non-nucleosides

Efavirenz and nevirapine did not show any clinically significant pharmacokinetic interactions with tipranavir/ritonavir. Minor reductions in nevirapine levels at a dose of tipranavir 500 mg and ritonavir

Table 1. Pharmacokinetic Parameter for Co-Administered Drug in the Presence of Tipranavir and Ritonavir

Study population/ co-administered drug	Co-admin. drug dose (mg)	Tipranavir/ ritonavir (mg bid)	<i>n</i>	AUC (%)	<i>C</i> _{max} (%)	<i>C</i> _{ph} (%)
Nucleosides						
<i>Healthy volunteers</i>						
Didanosine	400 qd	500/100	5	↔	↔	↔
Zidovudine	300 mg qd	500/100	29	↓43	↓61	↔
gZDV		750/200	25	↓33	↓56	↑25
		500/100	29	↔	↓18	↑52
		750/200	25	↑9	↓18	↑94
<i>HIV-1 positive patients</i>						
3-TC	150 bid	1250/100	35	↔	↓29	§
Abacavir		750/100	46	↔	↓14	§
		250/200	64	↔	↔	§
	300 bid	1250/100	11	↓35	↓52	§
		750/100	14	↓36	↓46	§
		250/200	28	↓44	↓44	§
D4T	30–40 bid	1250/100	19	↔	↓26	§
ddI		750/100	22	↓16	↓24	§
		250/200	26	↔	↔	§
	250–400 qd	1250/100	9	↔	↔	§
		750/100	8	↔	↔	§
		250/200	10	↓33	↓43	§
ZDV	300 bid	1250/100	23	↓31	↓51	§
		750/100	31	↓36	↓49	§
		250/200	48	↓42	↓46	§
Non-nucleosides						
<i>Healthy volunteers</i>						
Efavirenz	600 qd	500/100	24	↔	↔	↔
		750/200	22	↔	↔	↔
<i>HIV-1 positive patients</i>						
Efavirenz	1250/100	1250/100	15	↔	↔	↔
	750/100	750/100	19	↔	↔	↔
	250/200	250/200	23	↔	↔	↔
Nevirapine	1250/100	1250/100	17	24	↓29	23
	750/100	750/100	22	↔	↓14	↔
	250/200	250/200	26	↔	↔	↔

↓ or ↑: >30% change.
↔: <30% change and not statistically different from 1.00.
C_{pn}h: trough concentration measured at 24 h (EFV), 12 h (NVP, ddI), or 6 h (ZDV, gZDV).
§: trough concentration < limit of detection due to half-life of substrate drug.

200 mg are expected but these are not likely to be clinically significant and no dose modification is recommended. Adequate interaction studies with delavirdine have not been performed and therefore the use of delavirdine with tipranavir/ritonavir cannot be assessed.

G. Co-Administration of Tipranavir and Ritonavir with Protease Inhibitors

Pharmacokinetic studies of tipranavir and ritonavir with lopinavir, amprenavir, and saquinavir are being evaluated in trial BI 1182.51.

V. VIRAL RESISTANCE PROFILE OF TIPRANAVIR

A. Tipranavir Susceptibility of Clinical HIV Isolates

Viral susceptibility to tipranavir has been evaluated against two panels of protease inhibitor-resistant HIV-1 isolates. The tipranavir susceptibility of 105 HIV isolates was evaluated. The isolates selected had high levels of resistance to the available HIV protease inhibitors with more than a ten-fold phenotypic resistance to three or four peptidomimetic PIs and an average of 6.1 protease resistance mutations per isolate. The median EC_{50} to tipranavir (versus wild-type (WT) control) was 2-fold, compared to 46-fold for saquinavir, 44-fold for indinavir, 87-fold for ritonavir, and 45-fold for nelfinavir. Ninety-five isolates had tipranavir $EC_{50} \leq 4$ -fold WT, 8 had tipranavir EC_{50} of > 4 to ≤ 10 -fold WT and 2 isolates had tipranavir $EC_{50} > 10$ -fold WT (Figure 4).¹⁸

B. Development of Tipranavir Resistance *in vitro*

Development of tipranavir resistance *in vitro* has been quite difficult and slow when WT or drug-resistant HIV-1 was used to start the *in vitro* selection process. HIV-1 isolates NL4-3 (WT) and P37 (drug-resistant) were evaluated in serial passage in MT-2 cells in the presence of tipranavir. NL4-3 showed a 3.5-fold decrease in tipranavir susceptibility at passages 14 and 26. P37 virus showed a decrease in tipranavir susceptibility of up to 2.9-fold at passages 7, 13, and 22.¹⁹ No high level (> 10 -fold decrease in tipranavir susceptibility) resistance was detected in this study.

A WT HIV-1 isolate (pNL4-3 derived with 1.0 fold-sensitivity) was passaged in C8166 cells in the presence of increasing concentrations of

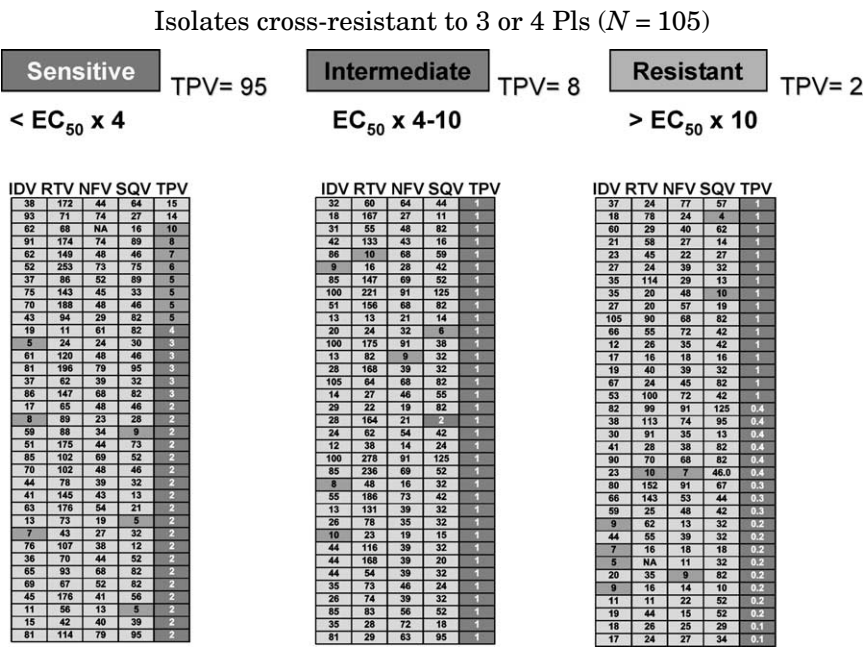


Figure 4. Summary of phenotypic testing by VIRCO.

tipranavir in tissue culture for 8 months.²⁰ Following each viral break-through the HIV-1 protease gene and adjacent cleavage sites were sequenced. Mutations emerged initially at positions L33F and I84V, producing a 1.7-fold decrease in tipranavir susceptibility. At passage 39, when the virus had 5 mutations and a 6.7-fold decrease in tipranavir susceptibility, a CA/p2 cleavage site mutation was detected. High-level resistance (14×) was not detected until virus broke through drug concentrations of 5 to 10 μM at passage 49. The virus with tipranavir resistance had mutations at positions: I13V, V32I, L33F, K45I, V82L, and I84V.

C. Development of Tipranavir Resistance in Trial BI 1182.2

In trial BI 1182.2 heavily protease-experienced NNRTI naive patients were treated with a regimen composed of 1 new NRTI, efavirenz, and tipranavir/ritonavir. Genotypic sequencing and phenotypic resistance testing using the VIRCO AntivirogramTM assay was conducted at baseline and attempted on all subsequent clinical samples containing HIV-1 RNA values > 400 copies/ml.

The 41 patients in the study had been exposed to an average of 2.5 protease inhibitors. At baseline the median EC_{50} to tipranavir (versus WT control) was 0.80-fold, as compared to 6.4-fold for saquinavir, 9.1-fold for indinavir, 18.6-fold for ritonavir, 24.1-fold for nelfinavir, and 1.7-fold for amprenavir (Figure 5). Forty HIV-1 isolates (97%) had $EC_{50} \leq 4$ -fold WT, none (0%) had an EC_{50} of > 4 to ≤ 10 -fold WT and 1 isolate (3%) had an $EC_{50} > 10$ -fold WT (Figure 6).

During the 48 weeks of the trial, 35 of 41 patients (85.4%) had either undetectable plasma virus or drug sensitive virus (< 4 -fold WT). Five

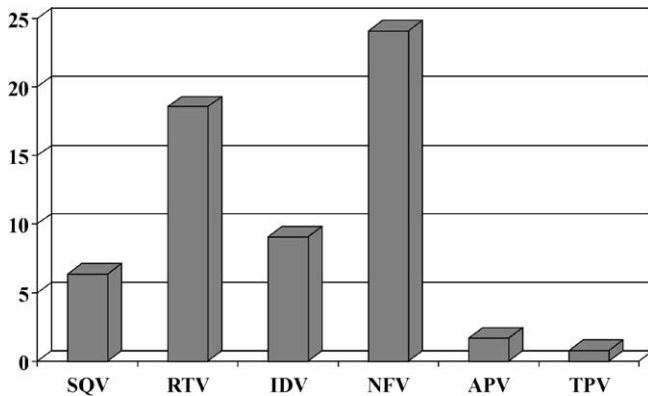


Figure 5. Baseline susceptibility (median fold-change) to peptidic PIs and tipranavir: trial BI 1182.2.

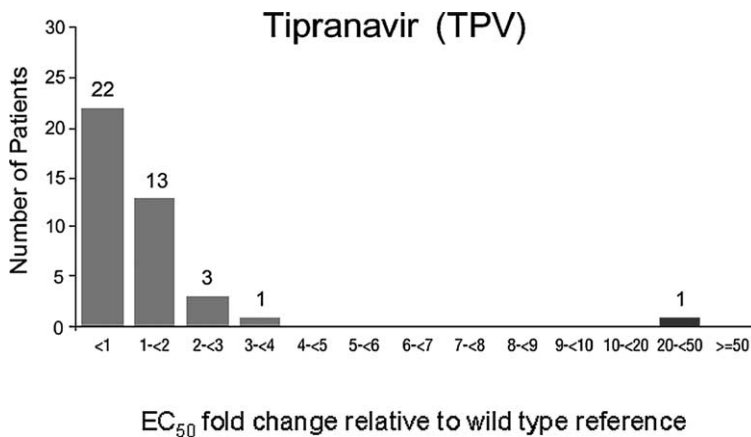


Figure 6. Phenotypic testing results at baseline for tipranavir: trial BI 1182.2.

patients (12.2%) had HIV isolates that developed decreased susceptibility to tipranavir (mean of 7-fold WT, range 4.6- to 10-fold WT). One patient who entered with tipranavir-resistant virus (23.7-fold WT) left the study prior to week 24 because of lack of efficacy with resistant virus (10.1-fold WT).

The 35 patients who had either undetectable or drug-sensitive virus at 48 weeks entered the study with a mean of 10.8 protease gene mutations. The 5 patients who developed decreased tipranavir susceptibility had a mean of 16.2 mutations at baseline. The patient who entered with tipranavir-resistant virus had 17 mutations at baseline. Factors associated with the development of decreased susceptibility to tipranavir were 16 or more protease mutations at baseline or prior exposure to all 4 available protease inhibitors. Five samples with decreased tipranavir susceptibility from patients who failed tipranavir treatment were obtained in trial BI 1182.2. The viruses from these heavily pre-treated patients had an average of 16 protease mutations at baseline. Comparison of baseline to subsequent viral isolates showed evidence of drug treatment selection at positions L33F/I/V and V82T. Viruses with reduced susceptibility to tipranavir that emerged during virologic failure on tipranavir had an average of 16 protease mutations with 3 of the following mutations: L33I/F/V, V82T, I84V, and L90M. The mutations associated with decreased susceptibility to tipranavir are shown in Figure 7.

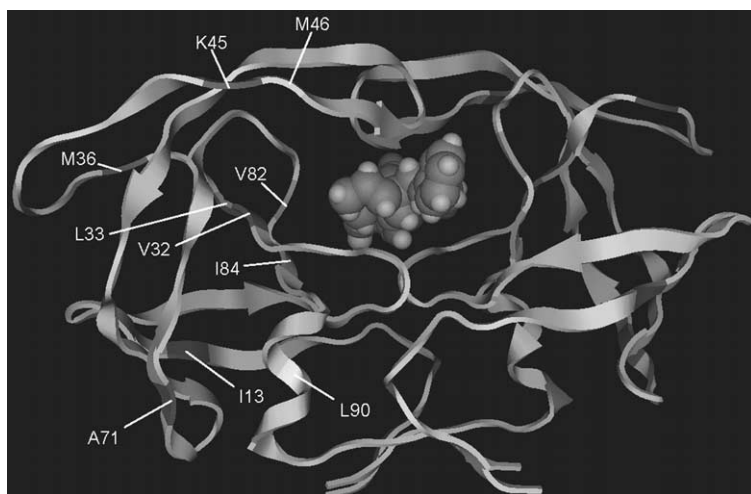


Figure 7. Location of tipranavir-selected mutations in HIV protease.

VI. CLINICAL TRIAL DATA—EFFICACY

A. Treatment of Antiviral-Naive Patients, Trial BI 1182.3

The antiviral efficacy of tipranavir (\pm ritonavir) has been studied in a two week, open label study in 31 treatment-naive, HIV-positive patients (trial BI 1182.3).¹²

Patients had plasma viral load (pVL) ≥ 5000 copies/ml and CD4 counts ≥ 50 cells/mm³. The dosing regimens, using a preliminary 300 mg SEDDS formulation, were:

- Group 1 ($n = 10$)—1200 mg tipranavir BID.
- Group 2 ($n = 10$)—300 mg tipranavir BID; 200 mg ritonavir BID.
- Group 3 ($n = 11$)—1200 mg tipranavir BID; 200 mg ritonavir BID.

Median decreases in pVL of 0.8 log₁₀ (Group 1), 1.4 log₁₀ (Group 2), and 1.6 log₁₀ (Group 3) copies/mL were achieved after two weeks of therapy (Figure 8). The CD4 cell count increased from a baseline median of 291 cells/mm³ by 11 (Group 1), 77 (Group 2), and 73 (Group 3) cells/mm³. Due to the suboptimal viral load response and high pill burden clinical development of un-boosted tipranavir was discontinued.

B. Treatment of PI-Experienced Patients—One Previous PI Regimen, Trial BI 1182.4

Preliminary 16 week data from an open label, parallel group study of ritonavir-boosted tipranavir (tipranavir/ritonavir) in single PI failure patients (trial BI 1182.4) have been presented.²¹

Patients were randomized to receive two new NRTIs plus tipranavir/ritonavir 500/100 mg bid ($n = 20$); or tipranavir/ritonavir 1250/100 mg bid ($n = 21$); or saquinavir/ritonavir 400/400 mg bid ($n = 21$). Tipranavir was given in the 250 mg SEDDS formulation.

There was unbalanced randomization at baseline: the median number of baseline protease mutations was higher in the tipranavir/ritonavir 500/100 mg group, 10.0 vs. 7.0 and 6.5 for the 1250 mg tipranavir group and the saquinavir group, respectively ($p = 0.004$); there was also a trend for higher baseline phenotypic resistance to PIs in the tipranavir 500 mg group in comparison to the other two groups.

By 16 weeks, the median pVL had decreased by 1.44 log₁₀ copies/mL in the low dose tipranavir/ritonavir arm; by 1.79 log₁₀ copies/mL in the high dose tipranavir/ritonavir arm; and by 1.75 log₁₀ copies/mL in the saquinavir/ritonavir (ITT, last observation carried forward analysis). There were no significant differences between the response rates in all

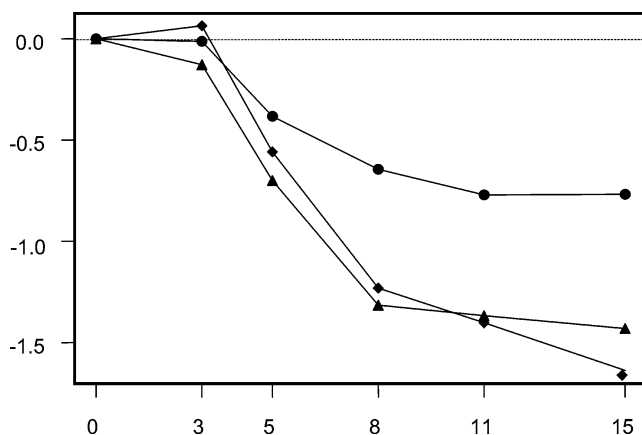


Figure 8. Median change in log HIV-1 RNA (< 50 copies/mL) in trial BI 1182.3. (●) Tipranavir 1200 mg; (▲) tipranavir 300 mg + ritonavir; (◆) tipranavir 1200 mg + ritonavir.

three treatment arms for both virological and immunological parameters.

Genotypic analysis of baseline viral isolates showed that 28/63 patients were infected with virus which carried no resistance mutations on the protease gene associated with reduced susceptibility to PIs. Hence, these data only provide limited information on the efficacy of tipranavir/ritonavir in patients who have failed PI-based therapy because of the development of mutations associated with reduced susceptibility to PIs. Analysis of data from the patients who were infected with PI-resistant virus is ongoing.

C. Treatment of PI-Experienced Patients—Two or More Previous PI-Based Regimens, Trial BI 1182.2

Forty one patients, who had failed at least two PI-containing regimens but were NNRTI naive and had one new NRTI available which they had not previously received, were enrolled in a randomized, open label study (trial BI 1182.2).²²

The study regimens were:

- Group A—efavirenz (600 mg QD); NRTI; tipranavir (1200 mg BID HFC initially; followed by 500 mg BID SEDDS); ritonavir (100 mg BID);

- Group B—efavirenz (600 mg QD); NRTI; tipranavir (2400 mg BID HFC initially; followed by 1000 mg BID); ritonavir (200 mg BID initially; followed by 100 mg BID).

Patients in group A and B were comparable in terms of baseline characteristics. At baseline, the mean pVL was 4.51 log₁₀ copies/mL in group A and 4.46 log₁₀ copies/mL in group B.

Initially, the HFC formulation of tipranavir was used in this study; patients switched to the SEDDS formulation when it became available. Different doses of tipranavir and, in group B, different doses of ritonavir—100 mg bid rather than 200 mg bid—were used when the SEDDS formulation was administered. Hence, patients switched formulations and dosages at different time points during the study.

The mean reduction in pVL by week 48 was 2.34 log₁₀ copies/mL in group A and 1.71 log₁₀ copies/mL in group B (ITT analysis) (Figure 9).

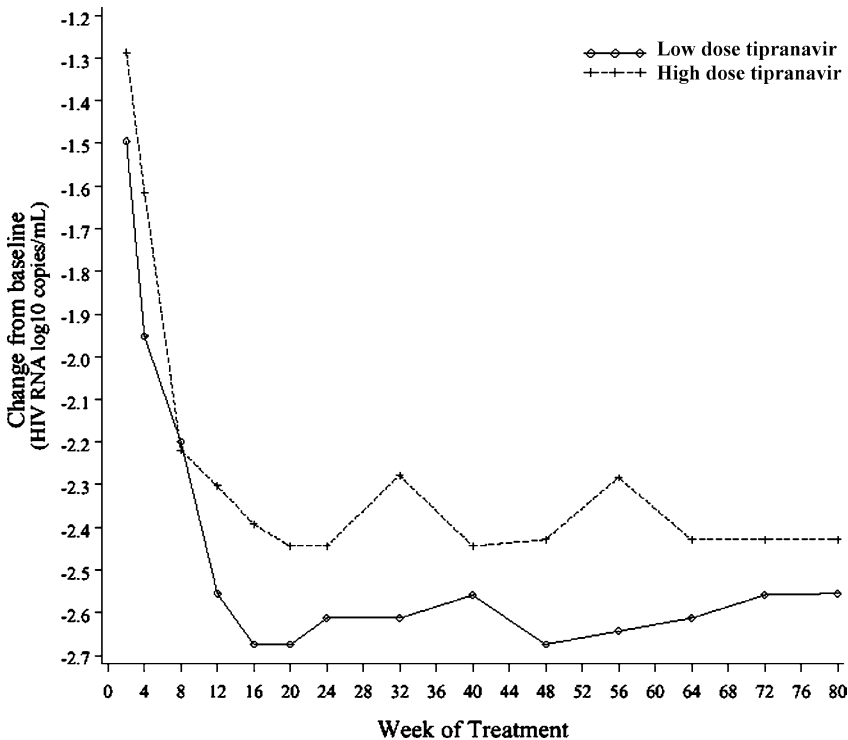


Figure 9. Median change from baseline in HIV-1 RNA values (log₁₀ copies/mL) for the Full Analysis Set (FAS) using Last Observation Carried Forward (LOCF) analysis: trial BI 1182.2.

78.9% of patients in group A had a pVL of < 400 copies/mL at week 48 (ITT MCF, missing considered failure analysis); percentage with pVL < 400 copies/mL in the corresponding AT-OC (as treated-observed cases) analysis was 93.8%. The majority of patients in group A had pVL < 50 copies/mL at week 48: 68.4% in the ITT MCF analysis and 81.3% in the AT-OC analysis. The response rates in group B were slightly lower, possibly because of sub-optimal adherence to the regimen and an increased incidence and severity of diarrhea in the high dose group. The mean reduction in pVL was similar, regardless of whether patients were infected at baseline with viral strains carrying > 5 or < 5 mutations associated with reduced susceptibility to PIs. Number of major PI mutations at baseline did not impact virological response; neither did the presence of any single specific PI mutation. There were four discontinuations (out of 10 in total) because of lack of

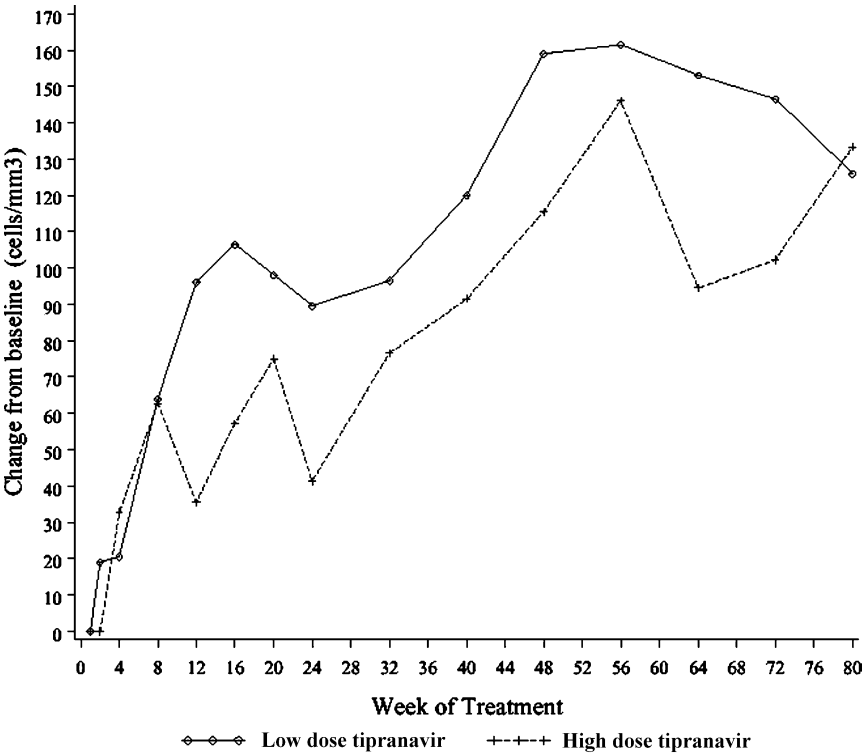


Figure 10. Median change from baseline in absolute CD4 + counts for the Full Analysis Set (FAS) using Last Observation Carried Forward (LOCF) analysis: trial BI 1182.2.

efficacy. Mean CD4 cell counts increased by 183.7 cells/mm³ in group A and 149.1 cells/mm³ in group B (Figure 10).

VII. CLINICAL TRIAL DATA—SAFETY SUMMARY

A. Treatment of Antiviral-Naive Patients, Trial BI 1182.3

Diarrhea (48%), nausea (23%), and fatigue (13%) were the most frequently reported drug-related adverse events. Most of the events (> 94%) were rated as ACTG grade 1 or 2 in severity. A total of three drug-related grade 3 adverse events (AEs) were reported by two patients. One patient in the tipranavir 1200 mg group reported grade 3 diarrhea and grade 3 vomiting. One patient, in the tipranavir 1200 mg + ritonavir 200 mg group, reported a grade 3 ALT elevation. No grade 4 AEs were reported during the 14-day tipranavir treatment period.

One serious adverse event was reported as grade 2 dizziness, it was not considered study medication-related. The patient withdrew from the study and fully recovered.

B. Treatment of PI-Experienced Patients—One Previous PI Regimen, Trial BI 1182.4

For all treatments, the most frequently observed AEs, regardless of causality, were nausea (41%), diarrhea (38%), fatigue (25%), and vomiting (23%). In addition, AEs, regardless of causality, occurring in ≥ 10% of patients in both tipranavir groups (combined data) consisted of headache (18%); pyrexia, upper respiratory tract infection (URTI) (16%); hypoesthesia, sinusitis, anorexia (12%); and rash (10%), rhinitis (10%). The frequency of diarrhea and other GI AEs was higher in the tipranavir 1250 mg/ritonavir 100 mg group (44%) compared with the tipranavir 500 mg/ritonavir 100 mg group (36%).

Thirteen patients had AEs which led to study discontinuation: 3 in the tipranavir 500 mg/ritonavir 100 mg group, 3 in the tipranavir 1250 mg/ritonavir 100 mg group, and 7 in the saquinavir/ritonavir group.

C. Treatment of PI-Experienced Patients—Two or More Previous PI-Based Regimens, Trial BI 1182.2

The most common adverse events in the study were gastrointestinal: diarrhea (59%)—15/24 mild; nausea (31%), vomiting (17%), however,

medication appeared to be well tolerated and only 2 discontinuations were because of adverse events (4).²² Other adverse events included rash, upper respiratory tract infection, fatigue, dizziness, insomnia, abnormal dreaming, increased sweating, increased ALT, GGT, sinusitis, and myocardial infarction.

VIII. SUMMARY

Tipranavir is a potent, highly selective, orally bioavailable, non-peptidic protease inhibitor (NPPI). It is specific for HIV protease with a median *in vitro* EC₉₀ value of 0.16 μ M against clinical HIV-1 isolates. The target plasma levels in man are based on a protein-adjusted EC₉₀ of 2.0 μ M for viruses with high level resistance to multiple protease inhibitors. Tipranavir suppresses the replication of laboratory and clinical HIV-1 strains in primary peripheral blood mononuclear cells.

Tipranavir acts additively or synergistically with other licensed antiretroviral (ARV) drugs, including other protease inhibitors (PIs). The majority (90%) of 105 HIV-1 isolates that were highly cross resistant to other PIs were sensitive to tipranavir when tested *in vitro*. The observed incidence of cross-resistance to tipranavir following treatment with currently available peptidomimetic PIs is low.

Co-administration of low dose ritonavir increases the AUC of tipranavir between six- and nine-fold. Interaction studies with tipranavir/ritonavir and NRTIs and NNRTIs have been completed. No dose adjustments are recommended when these agents are administered with tipranavir/ritonavir.

Treatment of PI-experienced patients with regimens containing tipranavir/ritonavir has been to control viral replication and improve CD4 cell count for more than 80 weeks. Tipranavir/ritonavir administration to antiretroviral-naïve patients resulted in decreases in plasma viral loads (pVL) of 1.4 to 1.6 log₁₀ copies/mL (median values) over a two week period. In highly treatment-experienced patients with extensive prior PI exposure treated with tipranavir/ritonavir plus efavirenz demonstrated a pVL reduction of 1.75 to 1.79 log₁₀ copies/mL (median values) over a forty-eight week period. In this study the majority of patients had either undetectable plasma virus or plasma virus that remained susceptible to tipranavir.

Tipranavir has demonstrated potent anti-retroviral activity both clinically and *in vitro*, and has a distinct and robust resistance profile. It is active both in treatment-naïve and highly treatment-experienced patients and has a generally well-tolerated safety profile.

IX. CURRENT DEVELOPMENT STATUS

Tipranavir is currently in Phase III clinical development. The confirmed dose for all Phase III clinical trials is 500 mg tipranavir with 200 mg ritonavir, taken together bid with or without food.

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DESIGN OF NEURAMINIDASE INHIBITORS AS ANTI-INFLUENZA VIRUS AGENTS

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I. INTRODUCTION

Influenza remains the major cause of mortality and morbidity among respiratory diseases.¹ The worst worldwide epidemic, or pandemic, on record struck in 1918 and the so-called “Spanish influenza” virus killed more than 20 million people, sometimes within hours after the first symptoms appeared. This disaster was followed by epidemics of Asian flu in 1957, Hong Kong flu in 1968, and Russian flu in 1977. The avian influenza virus that infected 18 Hong Kong residents in 1997 killed six people. Public health experts warn that another pandemic can strike any time now and that it could well be as vicious as the 1918 episode. Such an event will be caused by a “new” virus against which the human population has no immunity. With today’s crowded conditions and rapid transportation, the epidemic is expected to reach every corner of the globe. Millions of people will become ill, and many will die.²

There have been only two options available to reduce the impact of the influenza virus. The first one is vaccines. The influenza vaccines currently in use are inactivated subunit vaccines containing hemagglutinin (HA) and neuraminidase (NA) obtained from various strains of cultured flu virus. They are reasonably effective against the strain used to make the vaccine and are cost-effective. However, it would be

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difficult to make, test, and safety-test enough vaccine in time to protect many people against a new virus due to frequent antigenic shift in the viral surface proteins. It is doubtful whether vaccination would be useful in controlling an influenza pandemic, at least in the early stages. Such an exercise was, in fact, attempted in January 1976, when a swine flu outbreak occurred among army recruits at Fort Dix, New Jersey.³ It was thought that the 1918 "Spanish influenza" virus might have returned, prompting President Ford to authorize the expenditure of \$350 million to "vaccinate every man, woman, and child in the USA." This mass vaccination program experienced a number of problems: low antibody titer, vaccine side effects, and litigation tangles—that could happen again if such an exercise were ever to be repeated.

The second option and a promising first line of defense is antiviral drugs. However, until a few years ago, those available antiviral drugs, amantadine and rimantadine, were limited in their effectiveness because of their lack of activity against influenza B viruses, the rapid emergence of resistant viral strains and toxicity.^{4,5} NA has long been considered as potential antiviral target.⁶ There are 50–100 NA spikes per virion.⁷ It is believed that during the life cycle of the influenza virus, NA cleaves terminal sialic acid residues from glycoprotein receptor of viral HA, preventing the self-aggregation of newly formed virus particles at the cell surface and promoting the release of virus from the infected cells. Studies with a NA-deficient influenza virus have shown that the mutant virus is still infective but the budding virus particles form aggregates or remain bound to the infected cell surface, therefore, spread of viruses in the respiratory tract could be stopped.⁸ Thus, influenza neuraminidase has been regarded as an attractive target for the development of new drugs for influenza infections.^{9,10}

The early phase of research efforts aimed at identifying inhibitors of NA resulted in only limited success.¹¹ The most potent inhibitors exhibited modest activity against both enzyme and virus replication *in vitro*. They were not selective for NAs of other origins such as bacteria and humans. They also lacked any appreciable activity in influenza infection models *in vivo*. The major breakthrough came in 1990s when the high-resolution crystal structures of sialic acid (**3**) and the transition state analogue Neu5Ac2en (**4**) bound to influenza A and B NAs were obtained.^{12–14} The vital information provided for the researchers in the field the great opportunity to use rational drug design strategy and discover more potent and selective inhibitors of the influenza virus NA. This approach has lead to the development of several well-known NA inhibitors. Two of them, zanamivir (GG167, **6**)¹⁴ and oseltamivir (GS 4104, **8**)^{15,16} have been successfully introduced

into the marketplace. Meanwhile, newer NA inhibitors are still being developed. Peramivir (BCX-1812, **9**), is in phase III clinical trials. Scientists at Abbott laboratories have reported the discovery of A-315675 (**10**) and the compound appears to be in the stage of pre-clinical development. Since a number of reviews on this subject have been published,^{17–21} the research on the identification of BCX-1812 and A-315675 will be discussed and included in this review.

II. X-RAY CRYSTAL STRUCTURES OF NEURAMINIDASE

The X-ray 3-dimensional molecular structures of NAs from both influenza A and influenza B were solved to high resolution in the early 1990s.^{14,22} Since then, the structures of complexes of NA with various small molecule inhibitors bound to the active site have been determined and they are available from Brookhaven Protein Databank. There are a few common structural features among all influenza NAs. First, The NA active site contains several well-defined pockets which are large and quite rigid. All residues make direct contact with the substrate and are strictly conserved among influenza NAs. A particularly striking characteristic of the NA active site is that there are a large number of polar or charged groups, thus implying the potential utilization of polar–polar or electrostatic interactions in potent inhibitor design. Indeed, detailed analysis of NA and inhibitors complex structures indicates several critical contacts. For example, the negatively charged carboxylate group of sialic acid makes strong charge–charge interactions with a cluster of positively charged side chains of an Arg triad (Arg 118, 292, and most importantly, 371) of NA. Whereas the N-acetyl group of sialic acid located opposite to the carboxylate group, makes both polar and hydrophobic contact with Arg 152, Trp 178, and Ile 222. These anchoring forces help the projection of the scaffold of sialic acid at the NA active site, thus forming a basis for the introduction of additional interactions which could lead to more potent NA inhibitors.

A close examination of the NA active site can lead to the identification of a few major binding pockets based on the crystal structures. Pocket 1 is mostly formed by Glu 276, Glu 277, Arg 292, Asn 294. One would consider this pocket as highly polar in nature as it interacts with the glycerol moiety of sialic acid. The careful inspection of NA structures reveals a potential hydrophobic pocket which is not utilized by sialic acid to achieve the optimal binding. This pocket is also surrounded by some highly conserved residues Ile 222, Arg 224, and

Ala 246. As demonstrated later in the design of the parent compound of oseltamivir,¹⁵ the potential non-polar nature at this site has been explored very effectively to fully realize the efficient hydrophobic interaction which was not seen with either sialic acid or zanamivir. Another binding pocket is formed by Glu 119, Asp 151, Arg 152, Trp 178, Ser 179 Ile 222, and Glu 227. The acidic residues in this pocket is not utilized by C-4 hydroxyl group in sialic acid and therefore, could be explored to add a strong charge-charge interaction between NA and designed inhibitors. These analyses clearly indicate that significant improvements could be made to increase inhibitors binding affinity to the NAs.

III. NEURAMINIDASE INHIBITORS DESIGN— A TWO-SCAFFOLD APPROACH

The first NA inhibitor synthesized was Neu5Ac2en **4** with K_i in the micromolar range.²³ A careful comparison of the structures of NA complexed with either sialic acid **3** or **4** revealed that sialic acid binds the enzyme in a considerably deformed conformation because of the carboxylic acid moiety on sialic acid forming the strong ionic interactions with triarginyl cluster of Arg 118, 292, and 371. This binding mode is quite similar to the corresponding one found in the complex of Neu5Ac2en bound to NA. However, in the latter case, the double bond in the pyranose ring imposes a planar structure around the ring oxygen. The activity and structure feature of this compound are in agreement with the proposed transition state based on biochemical mechanistic studies (Figure 1). It has been proposed that the catalytic mechanism for the cleavage of sialic acid (**3**) from glycoconjugate (**1**) by NA proceeds through the oxocarbenium cation transition state (**2**). This theory has led to the successful design of the transition mimetic NA inhibitors based on dehydropyranose and cyclohexene scaffolds. These include zanamivir and oseltamivir.

However, during the more recent research, it has been noticed that the center six-membered rings such as pyranose and cyclohexene can be replaced by a five-membered ring scaffold as such that the main function groups maintain their strong contacts with the respective pockets discussed earlier. From the point of view of conformation similarity, a five-membered ring is very much like a puckered six-membered ring containing a double bond. This strategy has proved quite effective and resulted in the identification of peramivir (BCX-

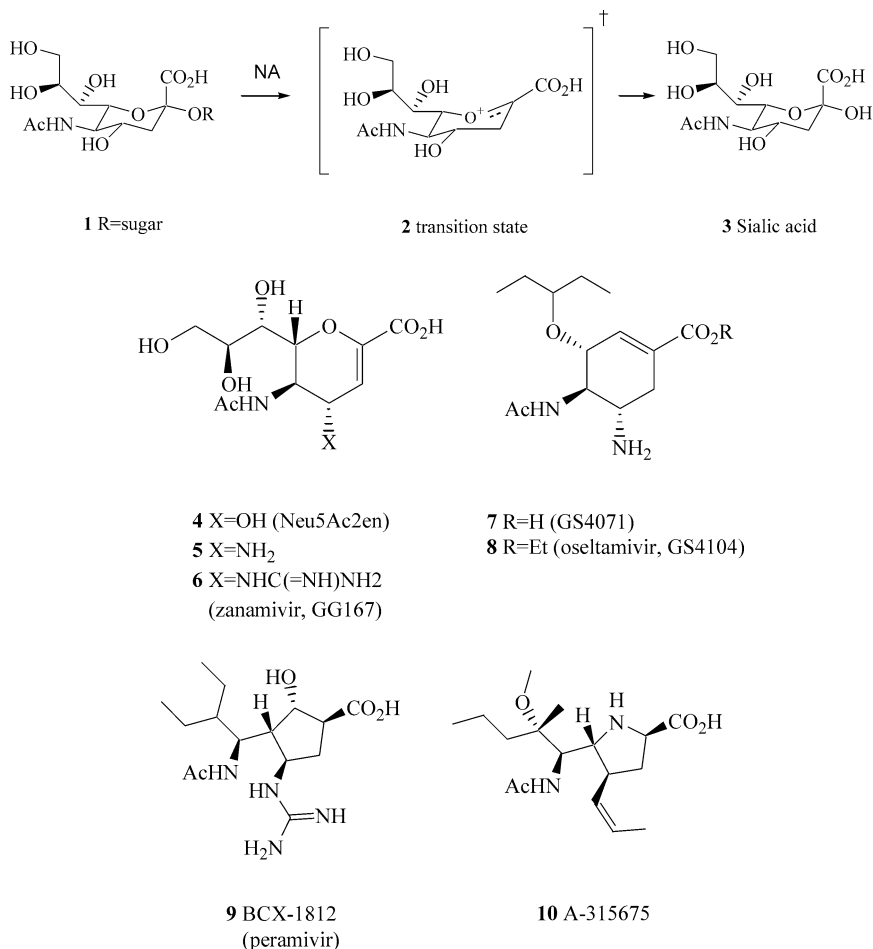


Figure 1. The catalytic mechanism of cleaving sialic acid from 1 and the lead NA inhibitors.

1812, **9**), a clinical development compound with a cyclopentane at the center, and A-315675 (**10**) containing a pyrrolidine ring.

IV. PYRANOSE-BASED INHIBITOR—ZANAMIVIR

As indicated before Neu5Ac2en (**4**) mimics the transition state of the cleavage of sialic acid glycoconjugate by NA to produce sialic acid. However, this compound proved to be a weak inhibitor and lacked both

selectivity and *in vivo* activity when tested in animals infected with influenza virus.²⁴ The structure of this compound bound to NA was determined later¹⁴ and the information on the binding mode provided the opportunity for rational design to improve the potency. Indeed, simply by replacing the hydroxyl group in Neu5Ac2en (**4**) with either an amino or a guanidino group, compound **5** and compound **6** were prepared and shown to be more potent NA inhibitors than compound **4** (K_i values: 10^{-8} M and 10^{-10} M, respectively).^{14,25} As expected, X-ray crystal structures revealed that the amino or guanidino group forms a salt bridge with Glu 119. On top of that, the guanidino group also generates multiple interactions with Glu 119, Asp 151 and Glu 227.¹⁴ However, the authors noticed that the hydrogen bonding of the guanidino group with Glu 119 is lacking because the carboxyl group of Glu 119 stacks parallel to the guanidino group.

A close inspection on the role of glyceryl side chain in **6** revealed its interaction with Glu 276 in a bidentate mode and stepwise truncations of this group compromised the activity (Figure 2) (**11** and **12**). However, more lipophilic replacement for the glyceryl chain led to the improved potency as seen in both compounds **13** and **14**. It is interesting to note that based on X-ray crystallographic studies, compound **13** binds to influenza A NA differently from influenza B NA.²⁶ It appears that the larger and rigid alkyl amide is less suitable for the influenza B NA active site.

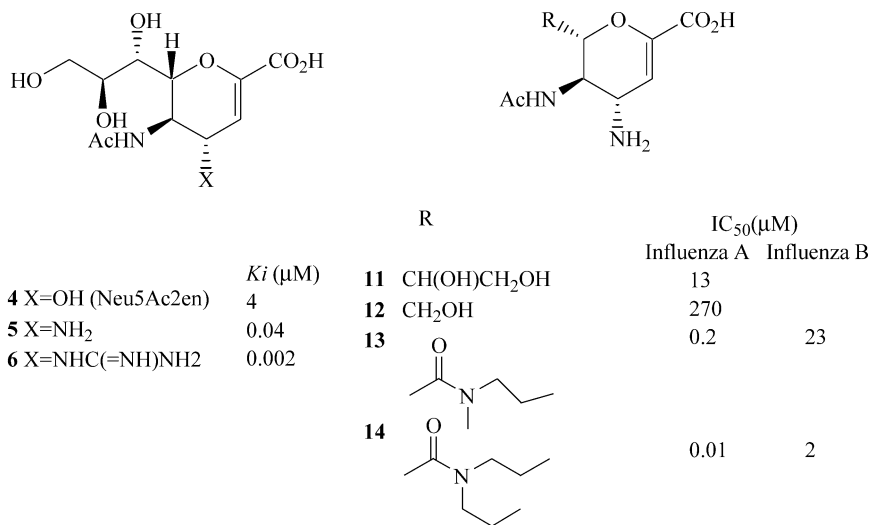


Figure 2. SAR of selected zanamivir analogues.

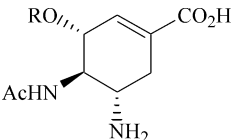

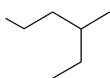
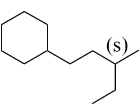
Zanamivir exhibited potent antiviral activity against a variety of influenza A and B virus strains in the cell culture assay, good selectivity for influenza neuraminidase as compared to other NAs such as the bacterial, para influenza and mammalian ones, as well as *in vivo* efficacy in animal influenza infection models by intranasal dosing.^{14,27} However, poor oral bioavailability and rapid excretion precluded its development as an oral drug for the clinical trials. Currently, zanamivir is being marketed in a formulation for inhalation.

V. CYCLOHEXENE RING-BASED INHIBITOR— OSELTAMIVIR (GS4104)

In the search for orally bioavailable and potent NA inhibitors, a new approach based on the cyclohexene scaffold was investigated.¹⁵ The double bond in the ring could be considered as an isostere of the oxo-carbonium cation of pyranose, therefore mimicking transition state **2**. Cyclohexene core should be stable both chemically and enzymatically. So a systematic medicinal chemistry program was executed. Examination of the crystallographic structures of sialic acid and its analogs bound to NA indicated that the C-7 hydroxyl of the glyceryl side chain does not interact with any NA active site residues. Hence, a synthetic strategy was adopted to replace the C-7 hydroxy methylene with an oxygen atom as a linker and a structure-activity relationship (SAR) was generated in a practical way (Table 1).²⁸ From the point of view of designing an orally bioavailable drug, any opportunity to achieve a balance between lipophilicity and solubility in water would be greatly appreciated.²⁹ The cyclohexene bearing all the polar groups making necessary strong contacts with NA active site residues described earlier is heavily hydrophilic. However, it is noticeable that sialic acid-NA complex structure contains certain lipophilic contacts between C-8 of the glyceryl side chain and the hydrocarbon chain of Arg 224.³⁰ The SAR shown in Table 1 strongly indicates that a reasonably large lipophilic group is preferred at that position since the activity increases as the size of the group becomes larger with the 3-pentyl (**7**) being the optimal for both influenza A and B NAs.

It is important to point out that there are two separate pockets to accommodate the R group of **15** and their shapes appear to be different between influenza A and B. The crystal structure of GS 4071 complexed with NA demonstrates that this series of inhibitors adopts a binding mode similar to that of sialic acid in its center scaffold.¹⁵ However, a truly amazing finding is the ability of both pockets to accept the

Table 1. SAR of GS4071 Analogues

<div style="text-align: center;">  <p>15</p> </div>			
	R	IC ₅₀ (nM)	
		Influenza A	Influenza B
16	H–	6300	–
17	CH ₃ –	3700	–
18	CH ₃ CH ₂ –	2000	5
19	CH ₃ (CH ₂) ₂ –	180	–
20	CH ₃ (CH ₂) ₃ –	300	215
21	CH ₃ (CH ₂) ₅ –	150	1450
7	(CH ₃ CH ₂) ₂ CH– (GS4071)	1	3
22	CH ₃ (CH ₂) ₆ 	1	4
23(S)	Ph 	0.3	70
24(R)		12	35
25		1	21 500
6	Zanamivir	1	3

lipophilic groups defined as ethyl branched at pent-3-yloxy side chain in GS4071 since one of the pockets is surrounded by the polar residues Arg 224 and Glu 276. The structure information clearly explains why. As compared to the binding mode of NA/sialic acid, the binding of the pentyloxy group of GS4071 to this site of NA creates a situation that Glu 276 adopts the other conformation by charge–charge interaction with Arg 224 to form a hydrophobic environment with a larger space. In the case of zanamivir, the strong interaction between glyceryl side chain and Glu 276 prevents the occurrence of binding mode seen with GS4071. This discovery has been utilized by other researchers as well later in the design of BCX1812 and A-315675. A similar observation

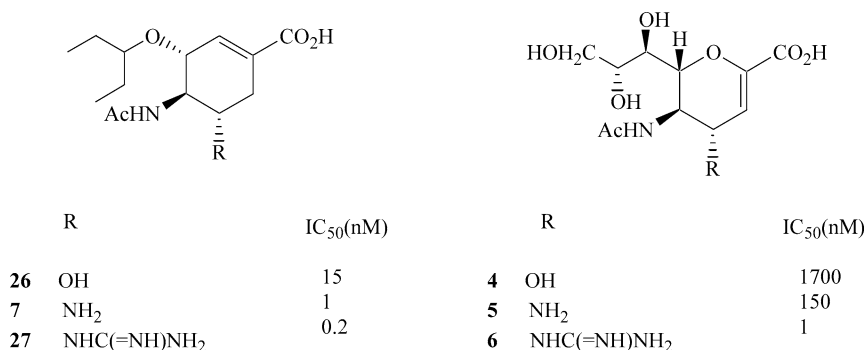


Figure 3. Comparison of the roles by R.

was made in studying a series of compounds represented by **13** and **14** (Figure 2).^{26,31} Replacement of the glyceryl side chain by a hydrophobic ether group can be viewed as a favorable development in achieving overall balance of polarity for a molecule to be used orally in clinic. A similar discovery was later made based on another hydrophobic interaction between Asp 151 side chain and the *Z*-propen-1-yl group in A-315675 (**10**) replacing the guanidino or amino group with maintenance of activity.^{32,33}

An interesting comparison of the role of C5-group in the cyclohexene series against zanamivir analogues was made. As seen from Figure 3, the replacement of the C-5 amino group in GS4071 by either a hydroxyl, or more interestingly, a guanidino group led to only modest to marginal changes in the activity. This implies that the significant hydrophobic interactions by the pentyloxy group at C-3 diminished relatively the importance of the guanidino group for the overall binding affinity. This can be regarded as a major advantage since in the oral drug design, it is widely believed that a highly polar guanidine function group would have a negative impact towards the permeability, hence, would generally compromise the oral bioavailability.

Finally, the cyclohexene ring itself was examined for the positioning of double bond.¹⁹ A systematic SAR study, as indicated in Figure 4, shows significantly reduced antiviral potency due to the double bond changes, suggesting the importance of a cyclohexene scaffold not only puckered, but also really mimicking the conformation of transition state during the NA catalyzed reaction. In earlier studies, it was found that a benzene ring replacing dehydropyranose seriously compromised activity even after introducing a guanidino group,³⁴ although it is in-

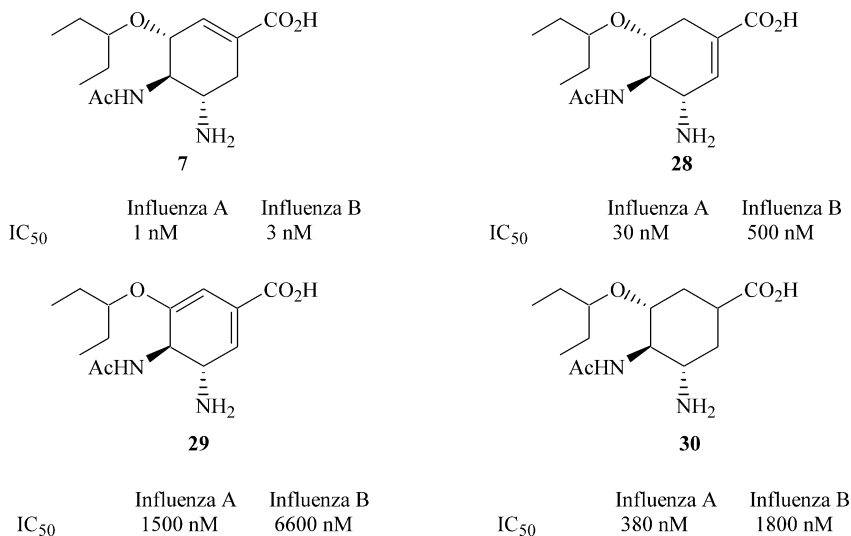


Figure 4. Effects of the cyclohexene scaffold structure.

teresting to note that the guanidino group binds to the pocket occupied by the glyceryl side chain of **4**.

In the pre-clinical evaluation, GS-4071 was found to be 5% bio-available by oral dosing. Nevertheless, conversion of the carboxylic acid to its ethyl ester, or oseltamivir (**8**), has significantly improved oral bioavailability in mice (30%), dogs (70%),¹⁶ and humans (75%).³⁵ A similar approach did not increase the oral bioavailability of zanamivir.¹⁶

VI. CYCLOPENTANE RING-BASED INHIBITOR— PERAMIVIR (BCX-1812)

It was reported in 1992 that a five membered furanose analogue (Figure 5) (**31**) and Neu5Ac2en (**4**) exhibited very comparable NA inhibitory activity.³⁶ As mentioned earlier, **4** mimics the intermediate **2** and hence has improved potency over sialic acid. Even though the central ring structure of compound **31** is different from that of **4**, the conformational superposition of NA complexes containing either **4** or **31** shows that the ring in **31** is significantly displaced from the pyranose ring of **4**.³⁷ Based on this observation, a cyclopentane scaffold was proposed by researchers at BioCryst Pharmaceuticals. Interestingly, the

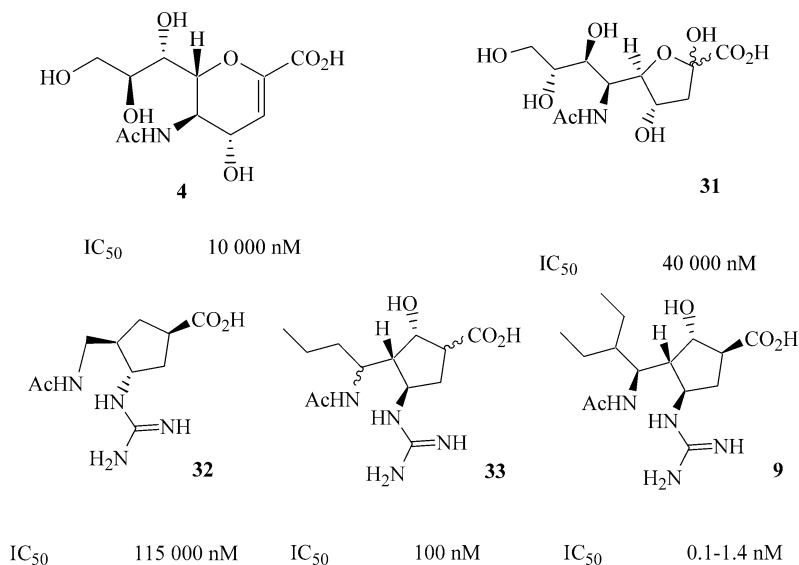


Figure 5. Identification of BCX-1812: a cyclopentane-containing NA inhibitor.

stereochemistry assignment of the lead isomers in each series in Figure 5 was obtained by X-ray crystal structures of inhibitor/NA complexes using soaking techniques. The guanidino group of peramivir **9** makes contacts with Asp 151, Glu 119, and Glu 227 in an altered way as compared to that of zanamivir.³⁶ It appears that this difference may account for the activity of **9** against a zanamivir resistant influenza A strain due to the Glu119Gly mutation.³⁸ As for the lipophilic pentyl group, BCX-1812 also induced the conformation change of Glu 276 in a manner similar to GS4071 (**7**)¹⁴ and compound **13**,²⁵ so as to create a hydrophobic pocket.

Despite the presence of the very polar guanidino and carboxylic acid groups, peramivir is orally effective in animal infection model studies and the current clinical trials are using once-a-day oral dosing schedule.

VII. PYRROLIDINE RING-BASED INHIBITOR—A-315675

The screening program at Abbott laboratories was initiated on a hypothesis formed upon analyzing the NA catalyzed reaction (Figure 1). In addition, in the transition state leading to the intermediate **2** there

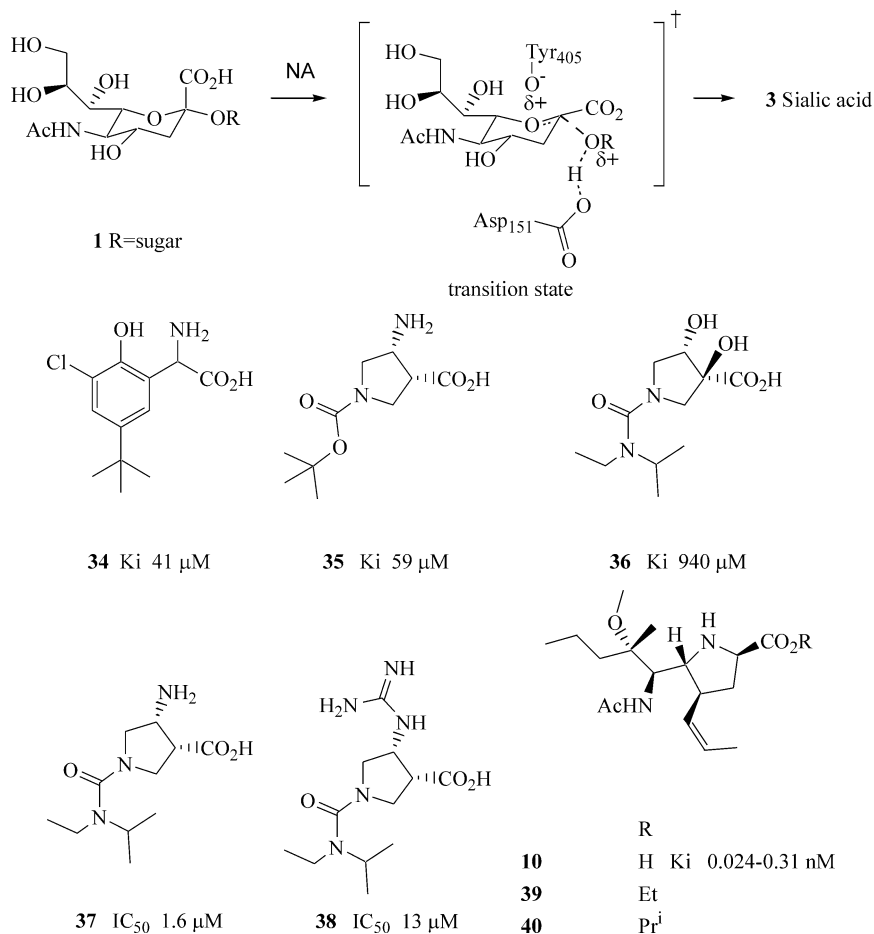


Figure 6. Discovery of A-315675.

is a positive charge developed at the glycosidic oxygen shown in Figure 6. This positive charge may be stabilized by an ionized Tyr 405.³⁹ It was proposed that an amino group could provide a positively charged species, thereby mimicking the transition state. Thus, a focused screening campaign using about 300 α - and β -amino acids was conducted and culminated in the discovery of A-315675 (**10**).⁴⁰⁻⁴² The two lead structures (**34** and **35**, Figure 6) from the screening were first analyzed by X-ray crystallographic studies of the compounds bound to NA.⁴⁰ The *t*-butyl group in **34** induced the conformation change of Glu 276 allowing the hydrophobic contact with the side chain of Glu 276 and

Arg 224. Compounds **36**, **37**, and **38** were designed to introduce a more favorable interaction between the corner hydroxyl group in **36** and Asp 151 and explore the role of the amino group as well as the hydrophobic interactions between dialkyl urea and the newly created pocket after reorientation of Glu 276.^{40,41} The interaction between the exocyclic amino group and Asp 151 is different from that seen with zanamivir since the replacement of amino by a guanidino group led to a small loss of activity, reminiscent of a small gain from **7** to **27** (Figure 3).

Upon further optimization by introducing other interactions between NA and inhibitor function groups such as acetamide present in zanamivir, oseltamivir, and BCX-1812, A-315675 (**10**) has been identified as a likely development candidate.⁴¹ Compound **10**, a substituted proline analogue, possesses the same stereochemistry on both the ring and the acetamido substitution as BCX-1812, suggesting a similar binding mode. Although it was mentioned that the X-ray crystal structure of A-315675 bound to NA shows no obvious changes to protein or inhibitor, and Glu 276 conformation change was observed as well, the exact structure information has yet to be published. What roles the ring amine, *Z*-propen-1-yl, and methoxy groups on the side chain are playing still remain speculative, though it is likely that the *Z*-propen-1-yl side chain makes the hydrophobic interaction with Asp 151 by the parallel stacking.³² A series of *in vitro* studies has shown that A-315675 is a potent inhibitor with sub-nanomolar activity against NAs of both influenza A and B. Furthermore, in a direct comparison to GS4071 (**7**) during the studies on the binding rate and equilibrium constants, particularly K_{off} , A-315675 exhibited the slow-binding or time-dependent inhibition similar to GS4071, but dissociation rates that were 12 to 18 times slower than for GS4071 with both influenza A and B NAs.⁴² It will be interesting to see whether this phenomena can be translated into a significant clinical benefit by once-daily dosing since the half life of dissociation for A-315675 is 10–12 h while data for both zanamivir⁴³ and GS4071 are calculated to be 0.55–1 h and these two agents are dosed twice-a-day.

Like GS4071, A-315675 has poor oral bioavailability in animal pharmacokinetic studies. Two pro-drugs of A-315675, A-315677 (**39**, the ethyl ester, Figure 6), and A-322278 (**40**, the isopropyl ester) were investigated.⁴⁴ Both compounds were rapidly hydrolyzed to A-315675 and the presence of A-315675 in lung interstitium was established in rats. The ethyl prodrug was further studied. The oral bioavailability of A-315675 derived from the ethyl ester prodrug was 50% in dog and the C_{max} of A-315675 was reached within one hour of dosing.⁴⁵

VIII. RESISTANCE TO NEURAMINIDASE INHIBITORS

There have been extensive reviews on the development of resistance to NA's inhibitors based on *in vitro*, *in vivo* studies, and clinical observations.^{17–21,46} Following serial passages of virus in cell culture in the presence of inhibitors and by genotypic analysis of the variants, it is known that mutations in both HA as well as NA regions can cause resistance to NA inhibitors. Clinically, there was only one documented case of resistance to zanamivir⁴⁷ and the rate of emergence of oseltamivir-resistant mutants does not exceed 1.3% in clinical trials (adults).³⁵ However, these mutants appear not to be cross resistant to other inhibitors and show reduced replicating capability and virulence as compared to the wild types.

From the point of view of structure changes, these inhibitors rely on the interactions with conserved residues differently from the natural substrate. For example, the guanidino group in zanamivir makes strong contacts with Glu 119 and Glu 227 whereas GS4071 utilizes the reoriented Glu 276 and these interactions do not occur with the natural substrate. Therefore, an E119G mutation causes 100 fold decrease in sensitivity of virus to zanamivir⁴⁸ but no change for GS4071.⁴⁹ An E119D mutant was selected with A-315675 after the first 8 passages *in vitro*.⁵⁰ As mentioned before, BCX-1812 retains its inhibitory activity against the zanamivir-resistant E119G variant of influenza A neuraminidase.³⁸ It is interesting to note that this mutation does not change the affinity for the substrate or the enzyme activity, but, rather, causes the enzyme tetramer to become unstable, resulting in low *in vivo* enzymatic activity. A second mutation occurs at Arg 292 (R292K). This causes 10 fold decrease of activity of zanamivir and 5000 to 30000 fold reduced sensitivity to GS4071.^{51–53} This is due to the ability of the mutant to alter the power of GS4071 to induce the conformation change at Glu 276.

Although HA mutations can be readily selected *in vitro* with most of NA inhibitors and appear to account for the majority of mutations identified in those studies, the *in vivo* consequences of HA mutations is not clear.^{52–55}

In vivo resistance studies in mice and ferrets have been conducted to evaluate the pathogenicity. The E119G mutant could not be clearly assessed due to a concomitant HA mutation of the virus studied.⁵⁶ All R292K mutants exhibited very poor infectivity in animal experiments.^{54–56} In addition, mutants have not been observed following passage of virus in mice under condition that would be expected to cause resistance to amantadine.⁵⁷ In general, NA mutants

might be compromised in fitting the natural setting. As to the HA mutations, although mutant isolates from oseltamivir treated patients have been obtained, none of them has been shown to be related to drug treatment or altered drug sensitivity.⁵⁸ One isolate with mutations in HA has been obtained from an immunocompromised patient receiving zanamivir.⁴⁷ The general significance of HA mutations in humans remains yet to be determined.

IX. *In vivo* ACTIVITY OF NEURAMINIDASE INHIBITORS AND CLINICAL STUDIES

All of four NAs inhibitors discussed have been evaluated in animal infection models for the efficacy studies. With the exception of zanamivir, all have been tested by oral administration of either prodrugs or the parent compound in the case of BCX-1812. Most of results have been reviewed recently.¹⁶⁻²⁰

In mice infected with influenza A or B virus, intranasal dosing of zanamivir or oral dosing of oseltamivir has been shown to be efficacious.^{59,60} Oral administration of oseltamivir also provided protection against the lethal effects of influenza A and B viruses infection in mice.^{60,61} In an experiment designed to determine the effect of delaying treatment with oral oseltamivir after infection, a 10 mg/kg/day dosage was applied for 5 days. By starting as late as 60 h post viral challenge with sufficient influenza A/NWS/33 (H1N1), oseltamivir was able to protect the mice from lethality, while 85% of untreated mice were killed.

Twice daily oral dosing of oseltamivir for 5 days beginning 4 h after infection was effective in the ferret model which has been thought to be more closely related to humans.⁶¹ A treatment by 5 and 25 mg/kg with oseltamivir was found to be sufficient to reduce the febrile response by 58 and 93%, respectively, in ferrets infected with a high dose of an influenza A/H3N2 virus. At both dose levels, peak virus titers were reduced in nasal wash samples. Previous experiments demonstrated that GS4071 level in bronchoalveolar lining fluid reached a C_{\max} similar to that in plasma, and showed long persistence.⁶²

BCX-1812,⁶³ when administered orally at 1 and 10 mg/kg, twice daily for 5 days, exhibited the efficacy comparable to or better than oseltamivir in 3 influenza A viruses and 1 influenza B virus infection models in mice. In the latter case, BCX-1812 appeared to provide better protection in terms of total survival rates. However, in another study, in ferrets, results were not quite as clear.⁶⁴

A-315675 ethyl or isopropyl ester prodrugs **39** and **40** have been shown to be equal to or more efficacious than oseltamivir in mice infected with influenza A or B virus.⁴² Whether these differences reflect the slower dissociation between NAs and the parent compound A-315675 remains to be seen.

Currently, intranasal zanamivir and oral oseltamivir are the only two NA inhibitors approved in many countries. The vast clinical data have been reviewed. Oseltamivir is available in both capsule and suspension formulations. Both are used in a twice-a-day, 5 days dosing regimen. Zanamivir is administered as a 10 mg/dose and oseltamivir is supplied as a 75 mg/dose. In blinded, placebo-controlled clinical trials, both oral oseltamivir⁶⁵ and intranasal zanamivir⁶⁶ have demonstrated efficacy in response to the treatment. Oseltamivir also reduced the level of inflammatory mediators (cytokines and chemokines) produced. Oseltamivir decreased the illness duration (76.3 and 74.3 h at doses of 75 and 150 mg, respectively, *versus* 97.0 h for placebo).

In a prophylaxis study, oseltamivir, when dosed at 75 mg once daily for 7 days, protected close contacts of influenza-infected persons against influenza illness, prevented outbreaks within households, and was well tolerated.⁶⁷ Likewise, intranasal zanamivir also exhibited the protection of healthy volunteers from experimental influenza infection when treatment was given prior to virus challenge.⁶⁶

BCX-1812 is still in clinical development and phase III clinical trials went on during the 2001–2002 influenza season in the USA.

X. CONCLUSION

The introduction of the novel NA inhibitors provided the mankind with the opportunity to control the influenza epidemics, a disease sometimes causing fatal consequences. The use of both oseltamivir and zanamivir in the clinic has proved that influenza NA is a valid target, and the significance of viral resistance is yet to be seen. These drugs will certainly find their wide use in both prophylaxis and treatment of human influenza virus infections.

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SIX-MEMBERED CARBOCYCLIC NUCLEOSIDES

Jing Wang, Matheus Froeyen, and Piet Herdewijn

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I. INTRODUCTION

Carbocyclic nucleosides have attracted the interests of medicinal chemists because of their inertness against the action of enzymes that cleave glycosidic bonds. Because of their relative metabolic stability, it is expected that these compounds have a longer half-life *in vivo*. The research on carbocyclic nucleosides with a five-membered ring has been very successful. Contrary, not much work has been done in the field of six-membered carbocyclic nucleosides (although the first articles covering this subject dated from the early sixties).

Here we give an overview of the compounds that have been prepared together with their synthetic schemes. These schemes cover a lot of different synthetic procedures and in this field, there is a lot of room for creativity in organic chemistry. Few of these compounds have been evaluated, and only a single example (cyclohexenyl G) is undergoing a more extensive exploration for its antiviral properties.

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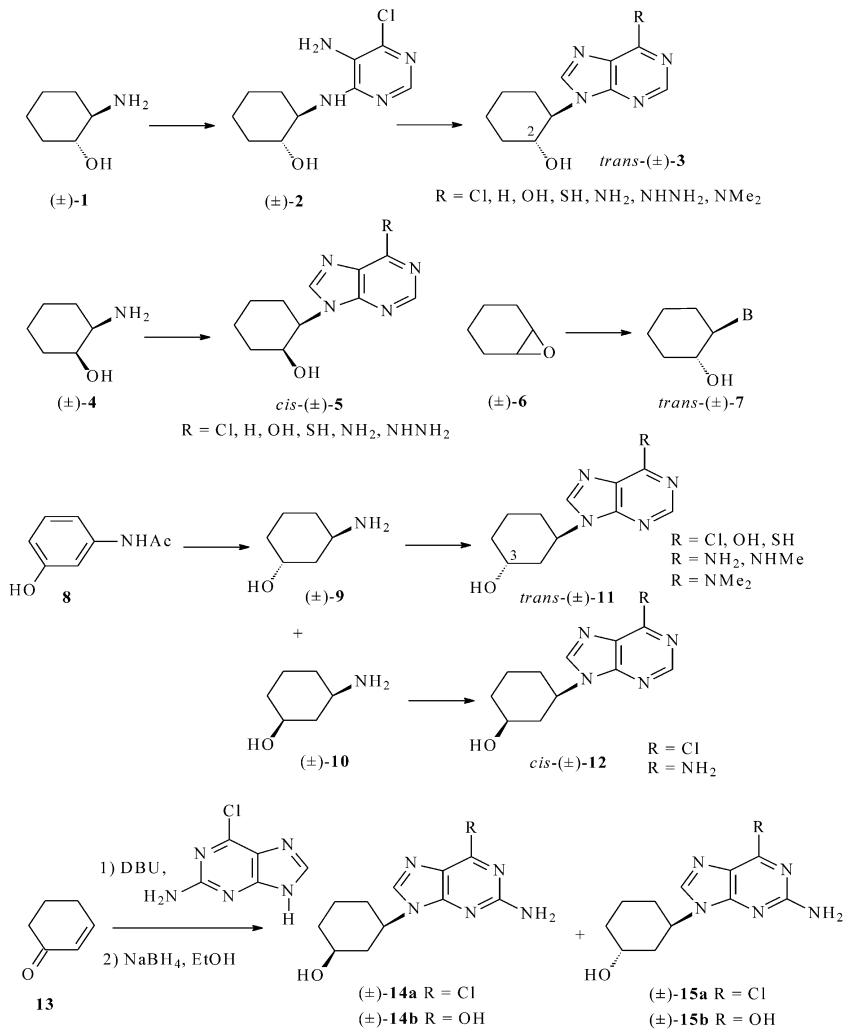
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II. SYNTHESIS OF CYCLOHEXANYL NUCLEOSIDES

A. Monosubstituted Cyclohexanyl Nucleosides

The first cyclohexanyl nucleosides were prepared by Schaeffer and co-workers in 1964.¹⁻³ These cyclohexanyl adenosine nucleosides have a substituent at either the 2, 3, or 4 position in a *cis* or *trans* orienta-

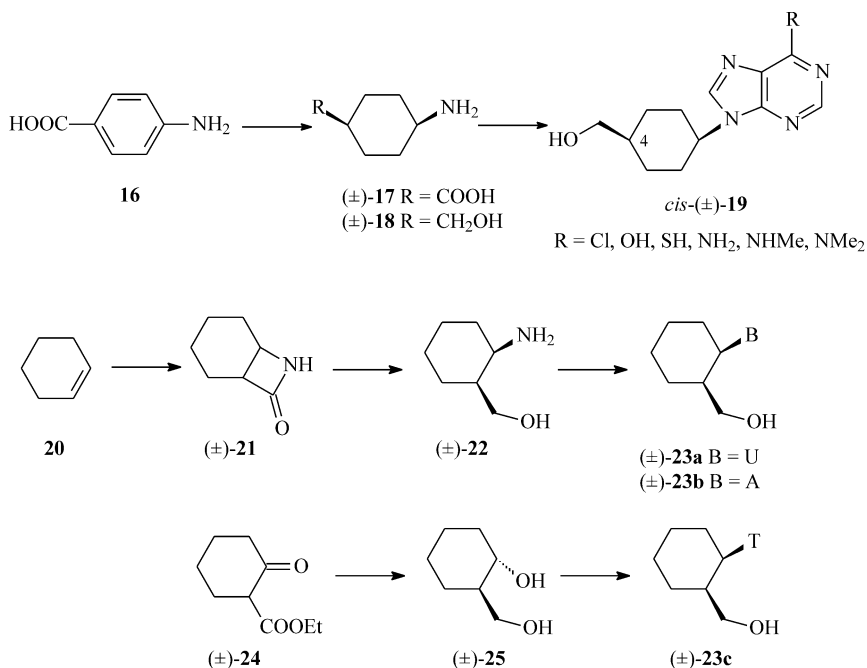


Scheme 1. Synthesis of monosubstituted cyclohexanyl nucleosides, part 1.

tion. The authors used the corresponding amino alcohol as starting material and the adenine base was constructed in a step-wise way (Scheme 1): i.e., condensation of the appropriate amino alcohol with 5-amino-4,6-dichloropyrimidine, followed by ring closure of the resulting substituted pyrimidine to give the desired purine. The 6-substituted purine analogues were obtained via nucleophilic displacement of the 6-chloro group. These monosubstituted cyclohexanols with an adenine base moiety or their 6-substituted purine analogues were tested as adenosine deaminase inhibitors. The *trans* 2-substituted nucleoside derivatives (\pm)-7 were prepared by opening of cyclohexene oxide with a nucleobase.^{4,5}

The 3-hydroxyl guanine derivatives **14** and **15** were synthesized by Michael addition of 6-chloroguanine to cyclohexenone, followed by reduction and separation of the *cis* and *trans* isomers.⁶

The *cis*-3-hydroxymethyl cyclohexanyl nucleosides were prepared from their cyclohexenyl congeners (see Section III, Scheme 12). The

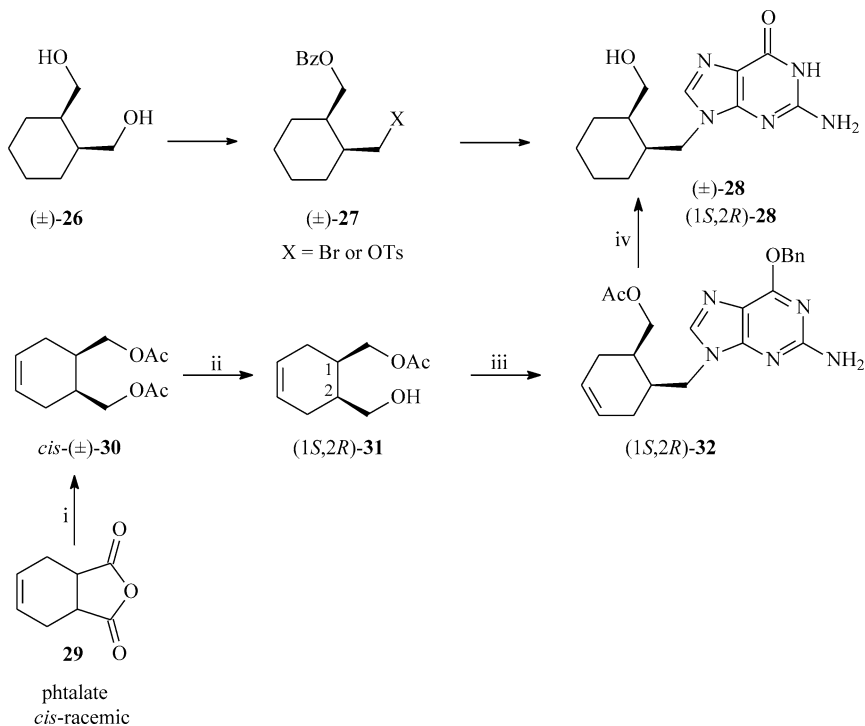


Scheme 2. Monosubstituted cyclohexanyl nucleosides, part 2: (i) (a) LiAlH₄, (b) AcCl, Py; (ii) PPL, pH = 7 buffer; (iii) (a) CBr₄, PPh₃, (b) NaH, 2-amino-6-benzoyloxypurine; (iv) (a) K₂CO₃, (b) 10% Pd/C, H₂.

cis-4-hydroxymethyl cyclohexanyl adenine and its 6-substituted purine derivatives **19** were prepared from *cis*-hydroxymethyl cyclohexanyl amine **18**, obtained by catalytic hydrogenation of *p*-aminobenzoic acid and reduction of the carboxylic acid group of **17** via its ethyl ester (Scheme 2).

The *cis*-2-hydroxymethyl cyclohexanyl uracil **23a** and adenine **23b** were constructed from the corresponding amino alcohol **22**.⁷ The latter was prepared in three steps from cyclohexene **20** by treatment with chlorosulfonylisocyanate, hydrolysis of the resulting lactam **21**, and reduction. The thymine derivative **23c** was synthesized by condensation of *trans*-(2-hydroxycyclohexanyl)methanol **25** with thymine using a Mitsunobu reaction.

The *cis*-(2-hydroxymethylcyclohexanyl)methyl guanine **28** (L-653180, Scheme 3) was prepared by a direct substitution of 2-amino-6-benzoyloxypurine on the mono-protected 1,2-cyclohexanedimethanol derivative **27**, followed by deprotection.⁸ The guanine compound (\pm)-**28**

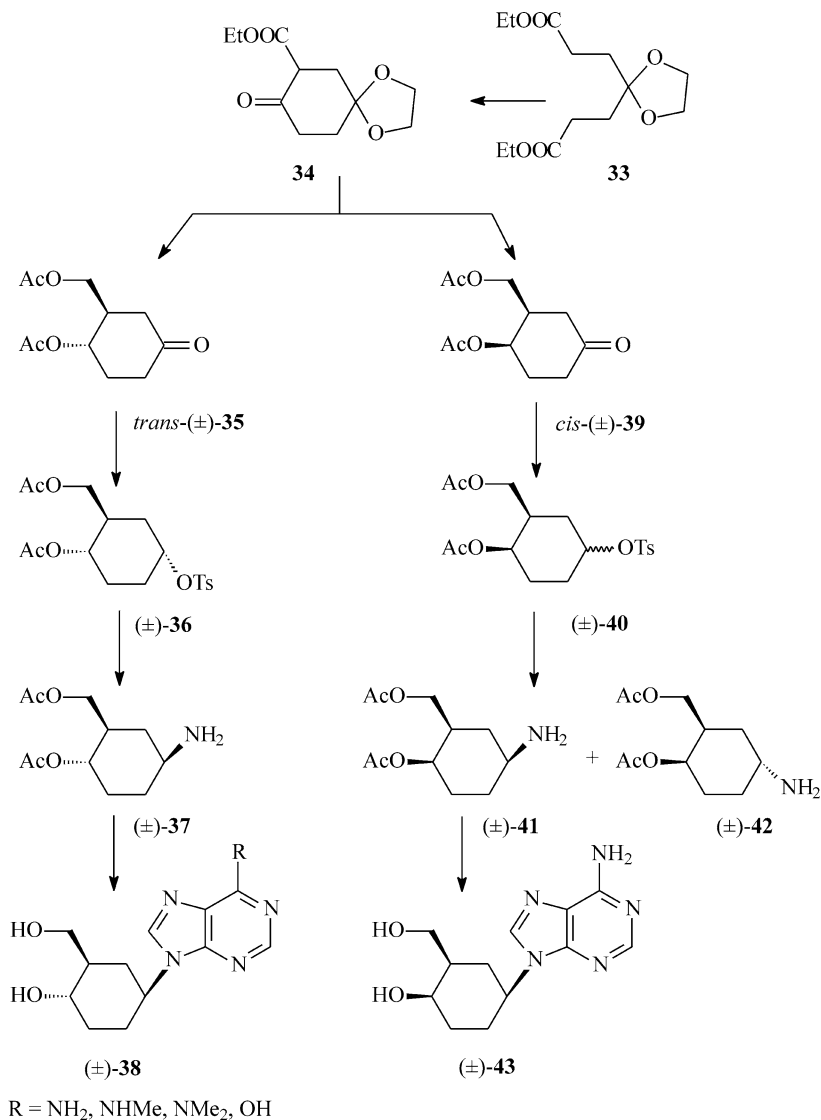


Scheme 3. Synthesis of monosubstituted cyclohexanyl nucleosides, part 3.

was found to be a selective inhibitor of HSV-1 thymidine kinase (TK).^{6,8-10} In order to determine differential activity of the enantiomers towards TSV-TK, L-653180 was resolved by chiral HPLC and the (1*S*,2*R*)-isomer was shown to be more active. Von Langen et al. reported an easy route to synthesize the (1*S*,2*R*)-enantiomer starting from inexpensive phthalate **29**.⁸ Reduction to the diol and treatment with acetyl chloride gave the *meso*-acetate **30**, which was selectively hydrolyzed with porcine pancreas lipase (PPL) to the chiral monoacetate **31**. Conversion of the free hydroxyl group into the corresponding bromide and coupling with the protected purine gave the acetate **32**, which was hydrogenated and deprotected to afford the (1*S*,2*R*)-enantiomer of L-653180.

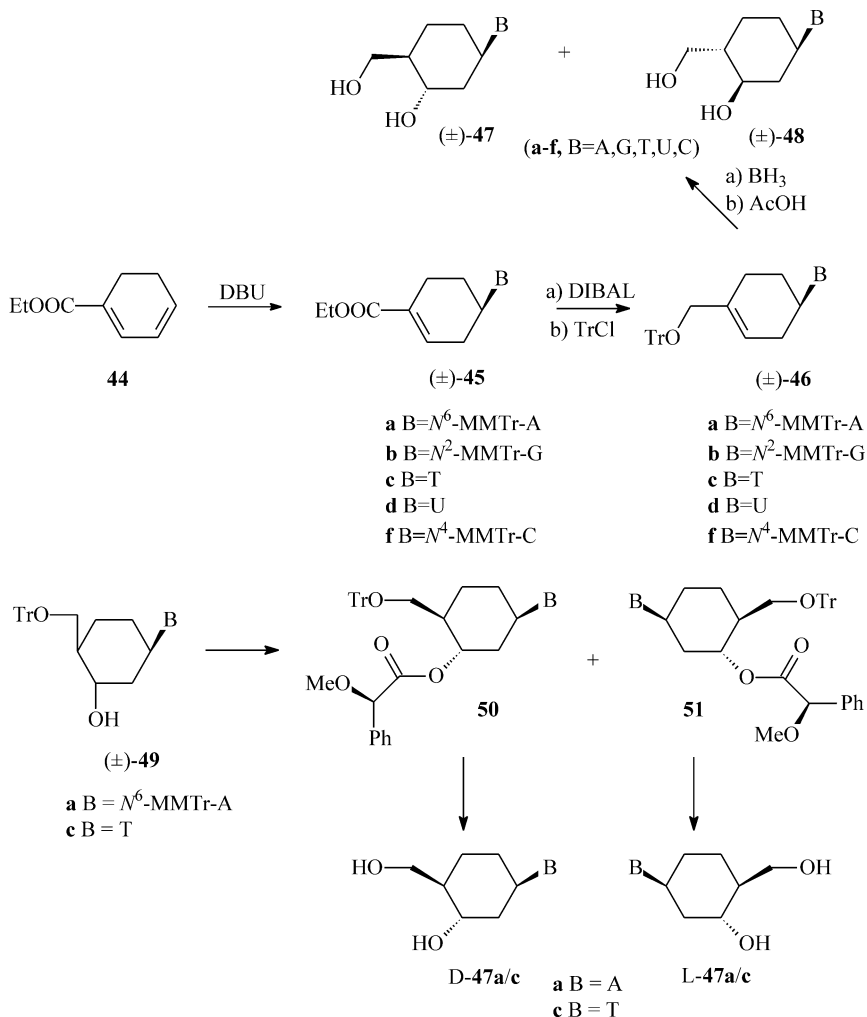
B. Di- and Trisubstituted Cyclohexanyl Nucleosides

During their studies of monosubstituted cyclohexane adenines as inhibitor of adenosine deaminase, Schaeffer et al. found that an OH group in position 2 increased inhibition compared to the unsubstituted one.¹ However, if an OH group was located at position 3 or 4, little change in inhibition was noted.^{2,3} It was concluded that the 2-OH group makes a contribution to binding to adenosine deaminase. In order to further study this effect, Schaeffer and Vince synthesized 4-hydroxy-3-hydroxymethyl-1-cyclohexanyl adenine and its 6-substituted purine analogs (Scheme 4).¹¹ Diethyl 4,4-ethylenedioxyphthalate **33** on Dieckmann cyclization gave 2-carbethoxy-4,4-ethylenedioxy-1-cyclohexanone **34**. Catalytic hydrogenation of the ketone followed by LiAlH₄ reduction of the ester gave 2-hydroxymethyl-4,4-ethylenedioxy-1-cyclohexanol, which after several additional steps led to separable *trans*- and *cis*-3-acetoxymethyl-4-acetoxycyclohexane **35** and **39**, respectively. Hydrogenation of the ketone group of **35** and **39** gave the alcohols that were converted into the tosylates **36** and **40**. Displacement of the tosylates with azide followed by catalytic hydrogenation of the azides gave the amines. The major product from **35** was 1β-amino-3β-hydroxymethyl-4α-hydroxycyclohexane **37**, whereas **39** gave a nearly equal mixture of 1β- and 1α-amino-3β-hydroxymethyl-4β-hydroxycyclohexanes **41** and **42**. Finally, the amines **37** and **41** were converted into 4α-hydroxy-3β-hydroxymethyl-1-cyclohexanyl adenine and its 6-substituted purine derivatives **38**, and 4β-hydroxy-3β-hydroxymethyl-1-cyclohexanyl adenine **43**. These compounds are weak inhibitors of adenosine deaminase.



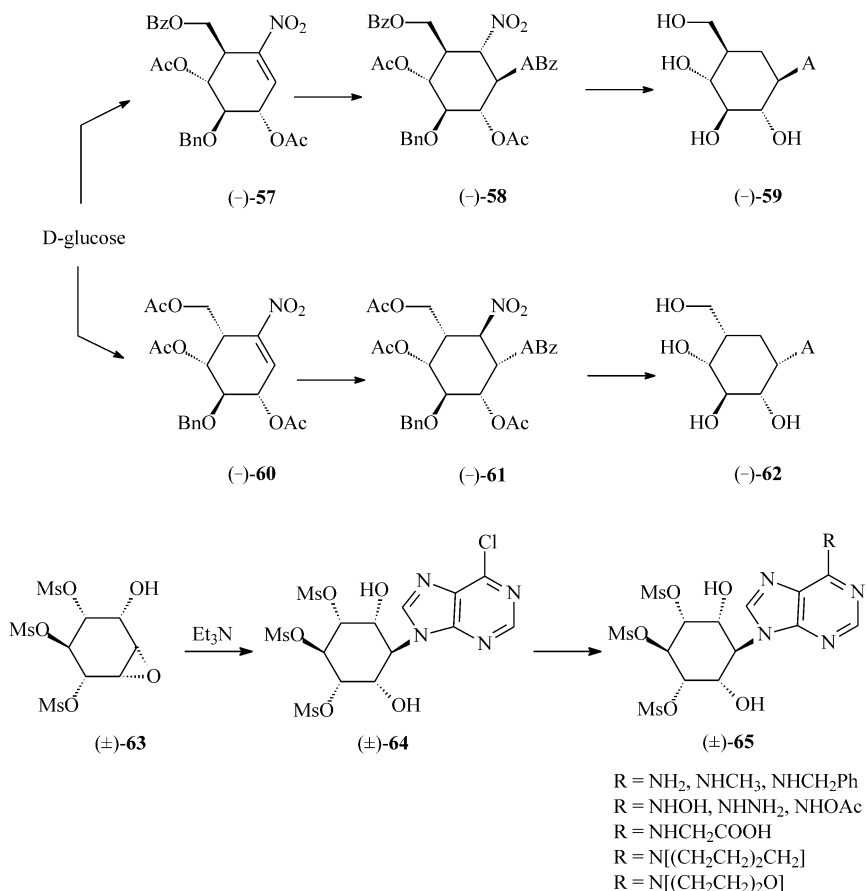
Scheme 4. Synthesis of di- and trisubstituted cyclohexanyl nucleosides, part 1.

The discovery that hexitol nucleosides showed antiviral activity stimulated the synthesis of their carbocyclic congeners, i.e., 3-hydroxy-4-hydroxymethyl-1-cyclohexyl nucleosides **47** (Scheme 5).¹²⁻¹⁴ 1,3-Cyclohexadiene-1-carboxylate **44** was used as starting material. The base



Scheme 5. Synthesis of di- and trisubstituted cyclohexanyl nucleosides, part 2.

moieties were introduced via a conjugated Michael-type addition using DBU as a base in DMF. Reduction of the ester group in **45** was accomplished using DIBAL to give the allylic alcohol, which was protected as trityl ether **46**. Further, hydroboration with BH_3 -THF gave a mixture of 1,4-*cis*- and 1,4-*trans*-isomers. Chromatographic separation of the two isomers and complete deprotection provided racemic 3 α -hydroxy-



Scheme 7. Synthesis of miscellaneous cyclohexanyl nucleosides.

9-pseudo- β -L-idopyranosyladenine **62** (Scheme 7).¹⁷ Reaction of **57** with *N*⁶-benzoyladenine in DMF in the presence of KF and 18-crown-6 gave the thermodynamically more stable adduct **58** having the adenine group in an equatorial position. Reductive elimination of the nitro group with *n*-Bu₃SnH and AIBN, followed by stepwise deprotection gave rise to (-)-9-[(1*R*,2*S*,3*S*,4*R*,5*R*)-2,3,4-trihydroxy-5-(hydroxymethyl)cyclohexyl] adenine **59**. The same procedure was applied for the synthesis of (-)-9-[(1*S*,2*S*,3*S*,4*R*,5*S*)-2,3,4-trihydroxy-5-(hydroxymethyl)cyclohexyl] adenine **62** starting from nitrohexene **60**.

Several 6-substituted purinyl-*muco*-inositol derivatives **65** were prepared by coupling triethylamine-activated 6-chloropurine with 2,3-

anhydro-1,5,6-tri-O-(methanesulfonyl)-*epi*-inositol **63** and substitution of the chlorine atom of **64** by nitrogen nucleophiles such as methylamino, diethylamino, benzylamino, hydrazino, morpholino, hydroxylamino, piperidino, and glycol groups.¹⁸

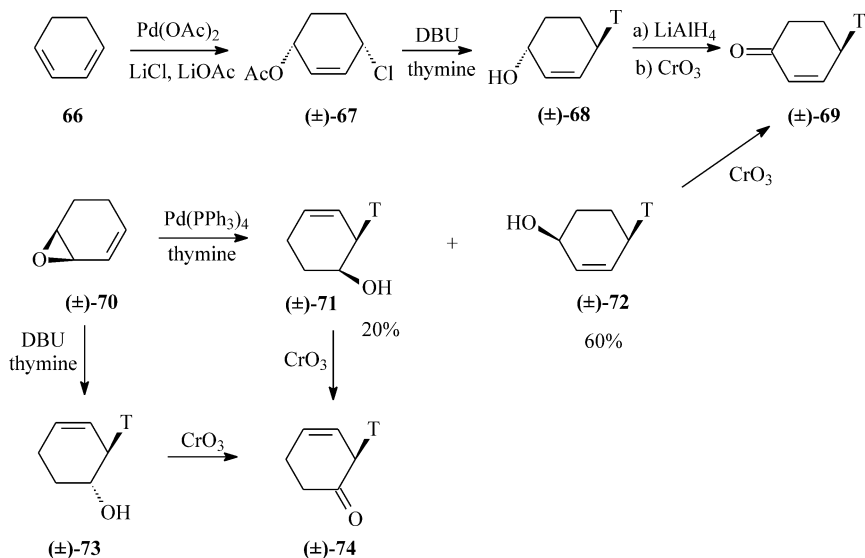
III. SYNTHESIS OF CYCLOHEXENYL NUCLEOSIDES

A. Monosubstituted Cyclohexenyl Nucleosides

1. Hydroxyl Group as Substituent

Several monosubstituted cyclohexenyl thymine nucleosides were synthesized by Arango et al. (Scheme 8).¹⁹ *Trans*-1-(4-hydroxy-2-cyclohexenyl) thymine **68** was prepared via Pd-catalyzed 1,4-chloroacetoxylation of 1,3-cyclohexadiene **66** to give *cis*-1-acetoxy-4-chloro-2-cyclohexene **67**, S_N2 substitution with thymine anion and hydride reduction. Jones oxidation of **68** gave 1-(4-oxo-2-cyclohexenyl) thymine **69**.

Addition of thymine to 3,4-epoxycyclohexene **70** in the presence of Pd(0) gave the *cis*-1-(4-hydroxy-2-cyclohexenyl) thymine **72** as the major product, as well as 20% of the regioisomer **71**. Direct nucleophilic ring opening of epoxide **70** with thymine in the absence of Pd(0) gave

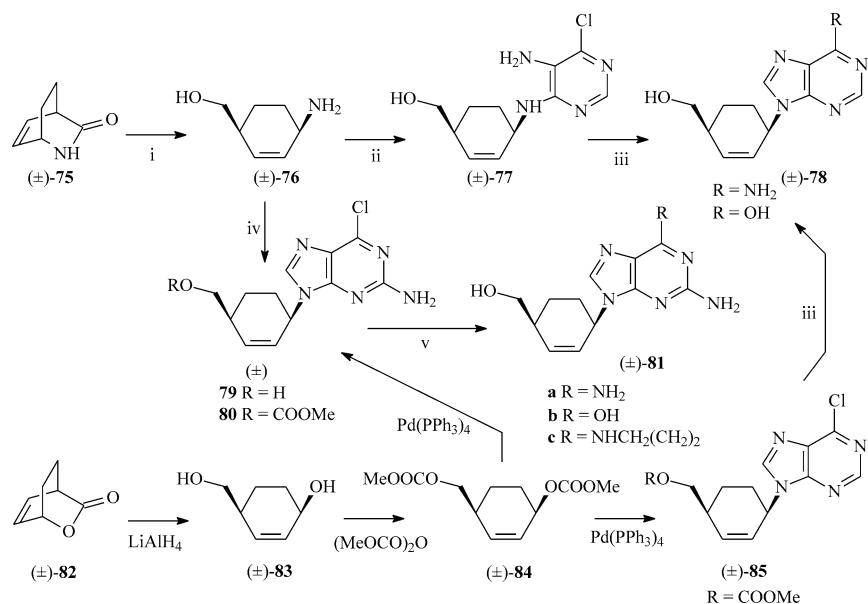


Scheme 8. Synthesis of monosubstituted cyclohexenyl nucleosides with a hydroxyl group as substituent.

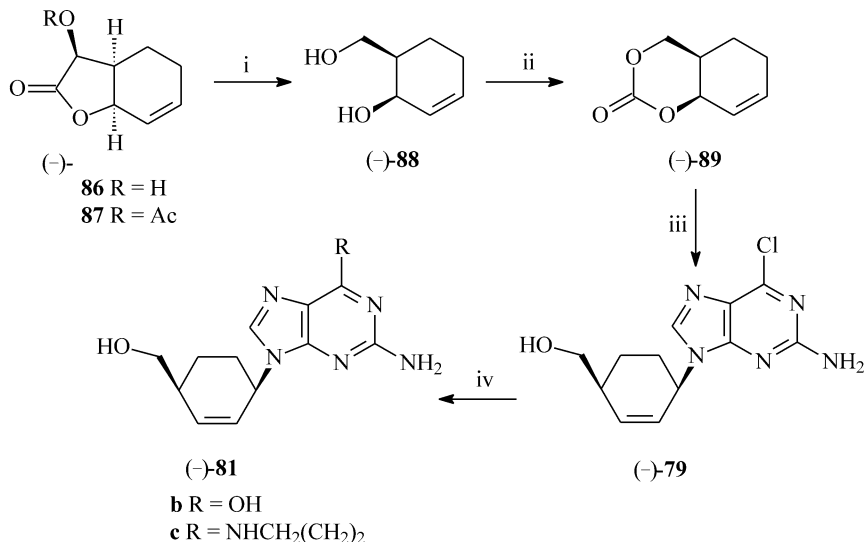
trans-1-(2-hydroxy-5-cyclohexenyl) thymine **73**. Oxidation of **72**, **71/73** gave the enones **69** and **74**, respectively.

2. Hydroxymethyl Group as Substituent

Carbovir and its derivative 1592U89 have been shown to act as potent antiviral agents.^{20,21} This discovery stimulated the synthesis of its cyclohexenyl analogs. Insertion of a methylene group between the carbons 4 and 5 of carbovir resulted in the cyclohexenyl analog of carbovir. Three approaches towards homocarbovir **81** were reported (Schemes 9, 10). The first two involved in a Diels–Alder reaction to construct the cyclohexenyl ring with the desired substituents, followed by stepwise construction of the base moiety or direct introduction of heterocyclic base via Pd(0)-catalyzed substitution. These two approaches generated 4-hydroxymethyl-2-cyclohexenyl nucleosides in a racemic form. The third approach provided optically pure homocarbovir via en-



Scheme 9. Synthesis of monosubstituted cyclohexenyl nucleosides with a hydroxymethyl group as substituent, part 1. (i) (a) (*t*-Boc)₂O, (b) NaBH₄, (c) TFA; (ii) 5-amino-4,6-dichloropyrimidine, DIEA; (iii) (a) (EtO)₃CH, HCl, (b) for **78a**, NH₃/MeOH; for **78b**, NaOH; (iv) (a) 2-amino-4,6-dichloropyridine, (b) 4-chlorobenzene diazonium hydrochloride, NaOAc, (c) Zn-AcOH, (d) (EtO)₃CH, HCl; (v) for **81a**, NH₃/MeOH; for **81b**, NaOH; for **81c**, cyclopropylamine, EtOH.



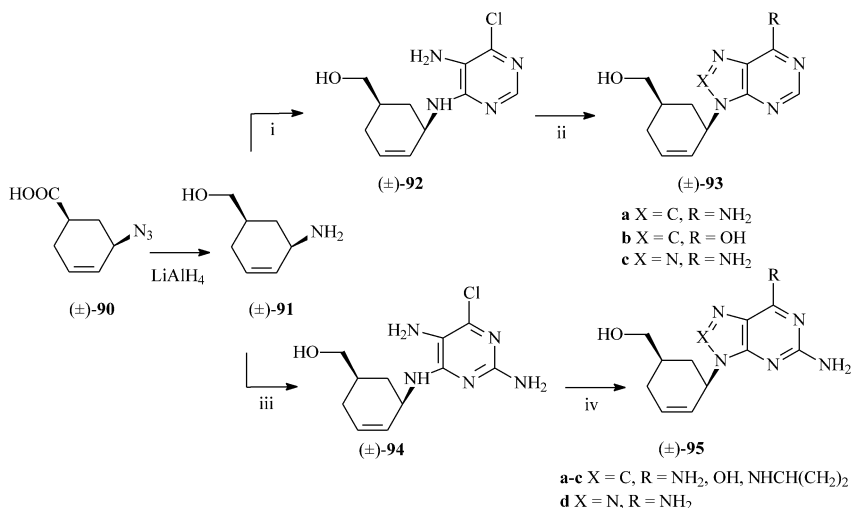
Scheme 10. Synthesis of monosubstituted cyclohexenyl nucleosides with a hydroxymethyl group as substituent, part 2. (i) (a) LiAlH₄, (b) NaIO₄, (c) NaBH₄, EtOH; (ii) triphosgene, Et₃N; (iii) 2-amino-6-chloropurine, Pd(PPh₃)₄, DMSO/THF.

zymatic resolution of an *endo*-hydroxylactone, followed by conversion to the bicyclic carbonate and subsequent Pd(0)-catalyzed introduction of the base moiety.

In Katagiri's method, the Boc-protected derivative of Diels–Alder bicycloamide adduct **75** (Scheme 9) was cleaved with NaBH₄ and the Boc-protecting group was removed under acidic conditions to generate the free amine **76**. Construction of the purine base from the amino function of **76** was carried out in a stepwise fashion via reaction with 5-amino-4,6-dichloropyrimidine or 2-amino-4,6-dichloropyridine to give **78** and **81**, respectively.²²

Konkel and Vince used a similar Diels–Alder reaction, i.e., 1,3-cyclohexadiene was reacted with chlorosulphonyl isocyanate to give the corresponding bicyclic lactone **82**. Reduction of the lactone afforded diol **83**, which was converted into its dicarbonate **84**. Palladium coupling with purine bases 6-chloropurine or 2-amino-6-chloropurine and hydrolysis gave rise to the desired nucleosides **78** and **81** with good regio- and stereoselectivity.²³

Olivo et al. started with *endo*-hydroxylactone **86** (Scheme 10).²⁴ Kinetic resolution using a lipase resulted in optically pure (-)-hydroxylactone **87**, which was converted in three steps into diol **88** in 75%

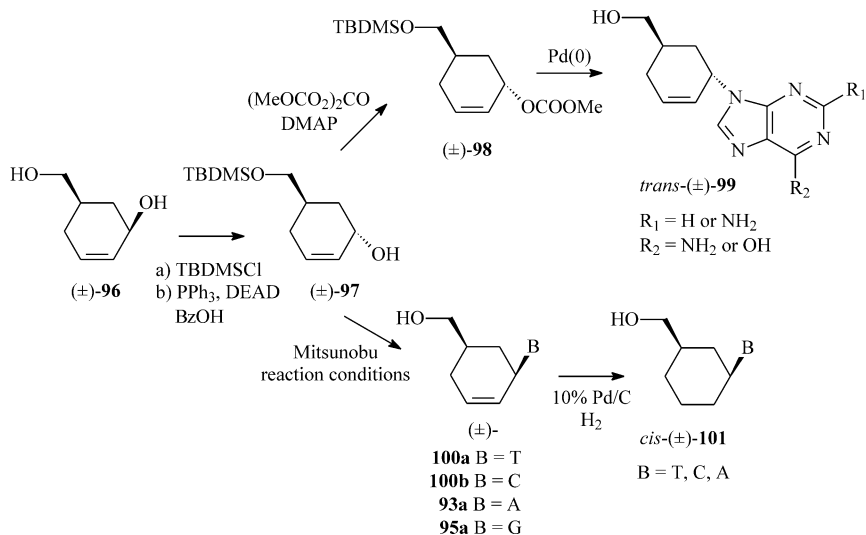


Scheme 11. Synthesis of monosubstituted cyclohexenyl nucleosides with a hydroxymethyl group as substituent, part 3. (i) 5-Amino-4,6-dichloropyrimidine, Et_3N ; (ii) (a) $(\text{EtO})_3\text{CH}$, HCl , (b) for **93a**, NH_3/MeOH ; for **93b**, NaOH ; for **93c**, HNO_2 then NH_3 ; (iii) (a) 2-amino-4,6-dichloropyridine, (b) 4-chlorobenzene diazonium hydrochloride, NaOAc , (c) Zn-AcOH , (d) $(\text{EtO})_3\text{CH}$, HCl ; (v) for **95a**, NH_3/MeOH ; for **95b**, NaOH ; for **95c**, cyclopropylamine, EtOH ; for **95d**, HNO_2 then NH_3 .

yield. Diol **88** was reacted with triphosgene and the resulting allylic bicyclic carbonate was coupled with 2-amino-6-chloropurine in the presence of a $\text{Pd}(0)$ catalyst. Further hydrolysis gave D-homocarbvir **81**.

Konkel and Vince synthesized *cis*- and *trans*-3-cyclohexenylcarbinol nucleosides. The *cis*-3-cyclohexenylcarbinol purine and azapurine nucleosides **93** and **95** (Scheme 11) were prepared using *cis*-3-azido-5-cyclohexenecarboxylic acid **90** as starting material. Reduction of **90** with LiAlH_4 gave rise to the amino alcohol **91** from which the purine and azapurine moiety was constructed in a stepwise manner to give the *cis*-3-cyclohexenylcarbinol nucleosides **93a–c** and **95a–d**.²⁵

The *trans*-3-cyclohexenylcarbinol nucleosides **99** were prepared starting from *cis*-3-hydroxy-5-cyclohexenylcarbinol **96** (Scheme 12).²⁶ The primary alcohol of **96** was selectively protected as its TBDMS ether and the secondary OH was inverted using a Mitsunobu reaction to give a *trans* allylic alcohol **97**. This was converted into its carbonate **98**, which was reacted with 6-chloropurine using palladium coupling methodology with retention of the configuration at C-1. Deprotection gave the 6-aminopurine derivatives, which were converted with adenosine deaminase to the guanosine and inosine analogs.



Scheme 12. Synthesis of monosubstituted cyclohexenyl nucleosides with a hydroxymethyl group as substituent, part 4.

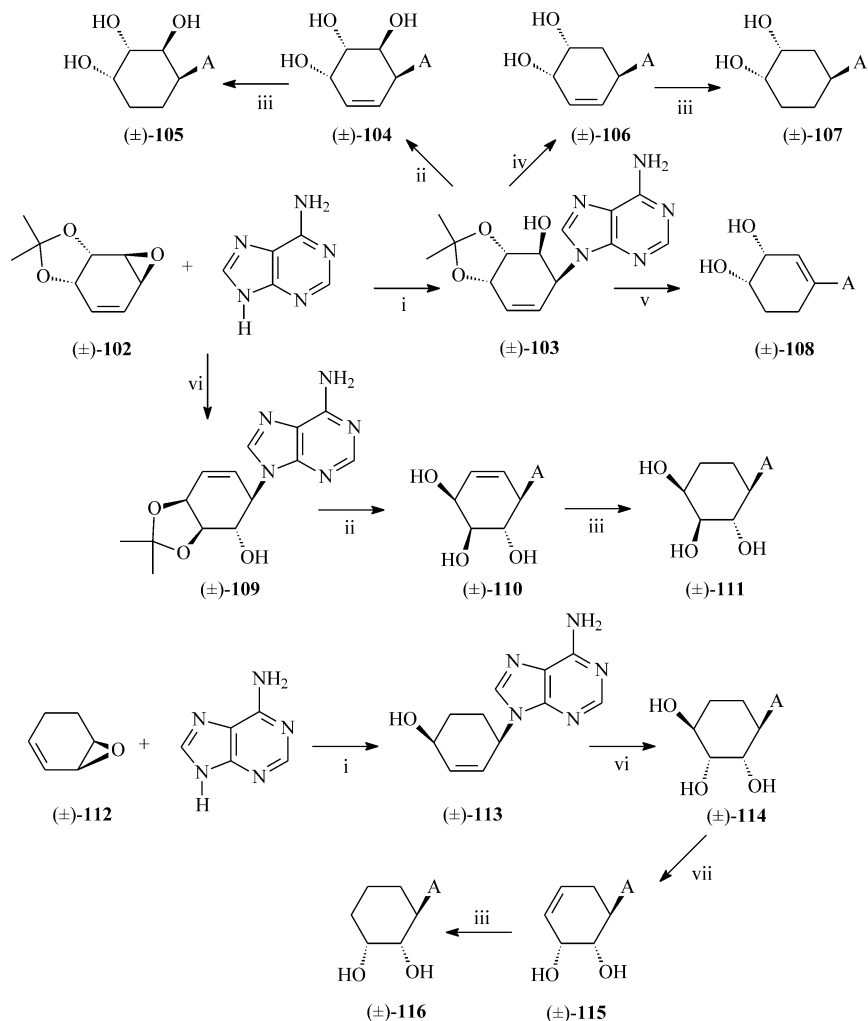
Recently, Barral et al. used the same intermediate **97** and applied a second Mitsunobu-type reaction to prepare all four *cis*-3-cyclohexenylcarbinol nucleosides **100**. Further, hydrogenation gave the corresponding cyclohexanyl analogs **101**.²⁷

B. Disubstituted Cyclohexenyl Nucleosides

1. Polyhydroxylated Cyclohexenyl Nucleosides

Several hydroxylated cyclohexenyl adenines were synthesized by Ramesh et al. as potential inhibitors of *S*-adenosyl-homocysteines (Scheme 13).²⁸ *Cis*-3,5-cyclohexadiene-1,2-diol as starting material was converted into allylic epoxide **102** via protection of the diol and selective mono-epoxidation. Pd(0)-catalyzed nucleophilic addition of adenine to **102** gave exclusively the 1,2-*cis* addition product **103**. In contrast, treatment of the allylic epoxide with adenine in the absence of a Pd(0) catalyst afforded the 1,2-*trans* product **109**. Both *cis*- and *trans*- products were converted into various di- and trihydroxylated cyclohexenyl- and cyclohexanyl adenines **104**, **105**, **106**, **107**, **108**, **110**, and **111**.

3,4-Epoxy cyclohexene **112**, obtained by mono-epoxidation of cyclohexadiene, was reacted with adenine in the presence of palladium to



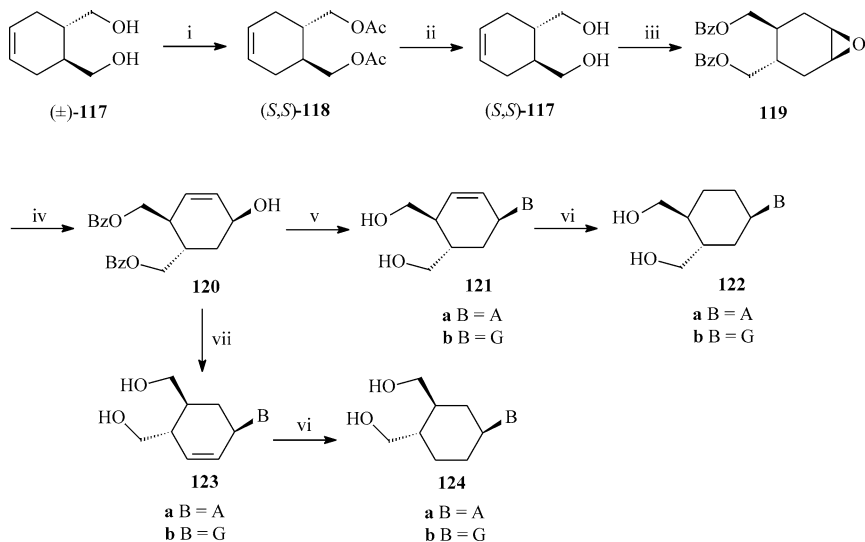
Scheme 13. Synthesis of polyhydroxylated cyclohexenyl nucleosides (i) [(*i*-C₃H₇O)₃Pl₄Pd, DMSO, THF; (ii) HCl; (iii) Pd-C, H₂; (iv) (a) *N,N*-dimethylacetamide dimethyl acetal, (b) *n*-BuLi/CS₂/MeI, (c) Bu₃SnH, AIBN, (d) HCl; (v) (a) PtO₂, H₂, (b) *N,N*-dimethylacetamide dimethyl acetal, (c) DAST, DMAP, (d) NH₄OH, (e) HCl, (f) Dowex-50W (H⁺); (vi) OsO₄, NMO; (vii) (a) DMP, HClO₄, (b) the same as (v) b–f.

afford the 1,4-*cis* product **113**. Oxidation with OsO₄ gave triol **114**. Protection of the *cis* diol, elimination with DAST and deprotection generated **115**, and via hydrogenation **116**.

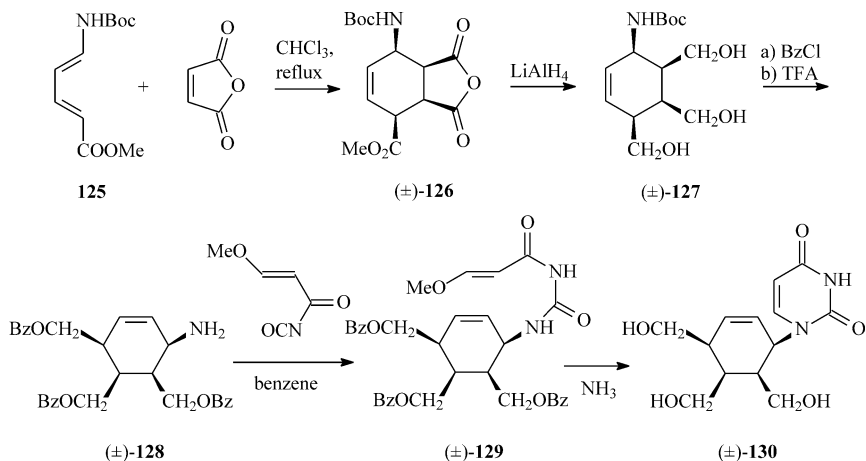
Except for **115**, which showed mild inhibitory activity against S-adenosylhomocysteine hydrolase, all hydroxylated cyclohexenyl adenines and hydroxylated cyclohexenyl adenines were devoid of inhibitory effects.

2. Di- or Trihydroxymethyl Cyclohexenyl Nucleosides

Enantiomerically pure di(hydroxymethyl) cyclohexenyl and cyclohexenyl purines were synthesized to be evaluated against HIV (Scheme 14). Rosenquist and Kvarnström resolved racemic *trans*-cyclohexene-4,5-diol **117** via *transesterification* using SAM-II lipase to obtain the optically pure diol (*S,S*)-**117**.²⁹ Epoxidation of the protected diol and treatment with TMSOTf and DBU followed by the acidic workup gave (1*R*,4*S*,5*R*)-4,5-bis[(benzoyloxy)methyl]-1-hydroxycyclohex-2-ene **120** in good yield. Pd(0)-catalyzed reaction of the acetate derivative of the allylic alcohol **120** with adenine and 2-amino-6-chloropurine followed by deprotection afforded the (1*S*,4*S*,5*R*)-4,5-dihydroxymethyl cyclohexenyl adenine and guanine derivatives **121a** and **121b**, respectively. Coupling of the allylic alcohol **120** with 6-chlo-



Scheme 14. Synthesis of di- or trihydroxymethyl cyclohexenyl nucleosides, part 1. (i) SAM-II, vinylacetate; (ii) NaOMe, MeOH; (iii) (a) BzCl, py, (b) *m*-CPBA; (iv) (a) TMSOTf, DBU, (b) H⁺; (v) (a) Ac₂O, py, (b) Pd(PPh₃)₄, NaH, adenine or 2-amino-6-chloropurine, (c) for **121a**, NH₃; for **121b**, NaOH; (vi) Pd-C, H₂; (vii) PPh₃, DIAD, 6-chloropurine or 2-amino-6-chloropurine, (b) for **123a**, NH₃; for **123b**, NaOH.



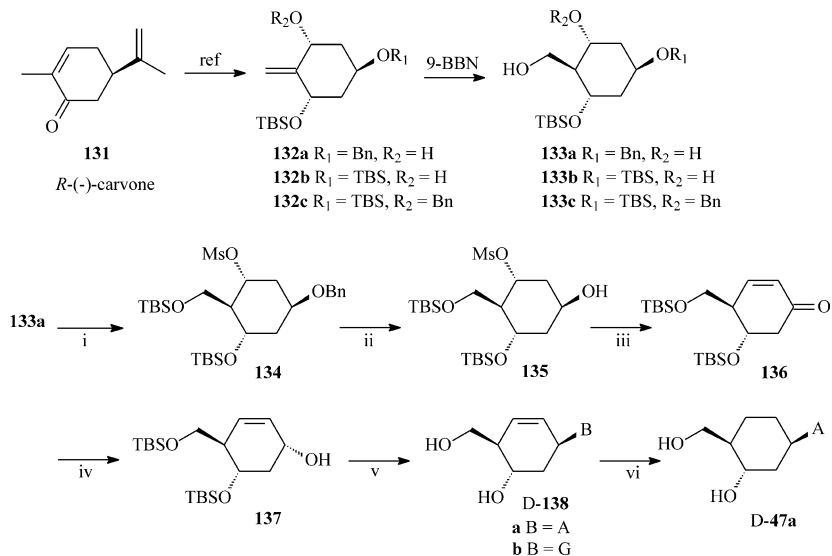
Scheme 15. Synthesis of di- or trihydroxymethyl cyclohexenyl nucleosides, part 2.

ropurine and 2-amino-6-chloropurine using a Mitsunobu procedure gave the corresponding (1*R*,4*S*,5*R*)-4,5-dihydroxymethyl cyclohexenyl adenine and guanine **123a** and **123b**, respectively. Hydrogenation of the cyclohexenyl nucleosides **121** and **123** gave the corresponding cyclohexanyl adenine and guanine compounds **122** and **124**.

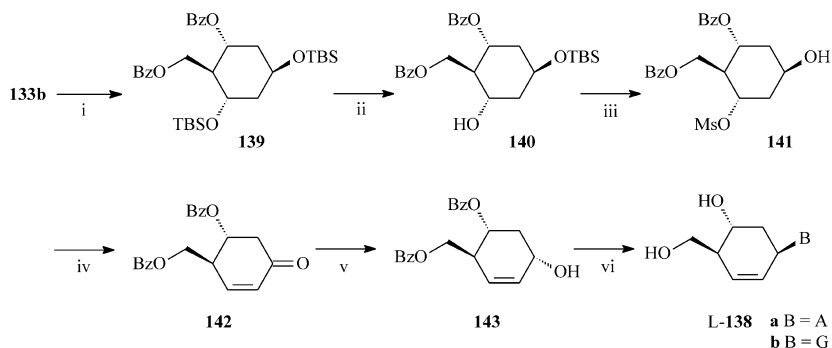
Racemic *cis*-2,3,4-trihydroxymethyl cyclohexenyl uracil **130** was synthesized via a Diels–Alder reaction between (2*E*,4*E*)-5-(*tert*-butoxycarbonylamino)penta-2,4-dienoic acid methyl ester **125** and maleic anhydride (Scheme 15).³⁰ Reduction of the adduct **126** using LiAlH₄ generated the triol **127**, which was protected as its benzoate. Removal of the Boc group yielded the free amino function from which the uracil moiety was constructed.

3. (D)- and (L)-3-Hydroxy-4-hydroxymethyl Cyclohexenyl Nucleosides

Wang et al. developed an enantioselective approach to the synthesis of both (D)- and (L)-3-hydroxy-4-hydroxymethyl cyclohexenyl nucleosides **138** starting from inexpensive chiral (*R*)-carvone (Schemes 16 and 17). These cyclohexenyl nucleosides can be considered as bioisosteres of natural furanose nucleosides. Simply speaking, the double bond of a cyclohexenyl nucleoside replaces the ring oxygen atom of a furanose and consequently the anomeric effect of a furanose is replaced by $\pi \rightarrow \sigma_{\text{C1}'-\text{N}}^*$ interaction between the double bond and the heterocyclic aglycon. The two stable half-chair ³H₂ (3'-endo) and ²H₃ (2'-endo) con-



Scheme 16. Synthesis of (D)- and (L)-3-hydroxy-4-hydroxymethyl cyclohexenyl nucleosides, part 1. (i) (a) TBSCl, imidazole, (b) MsCl, Et₃N; (ii) Pd-C, HCOONH₄; (iii) MnO₂; (iv) NaBH₄, CeCl₃·7H₂O; (v) (a) PPh₃, DEAD, adenine or 2-amino-6-chloropurine, (b) TFA/H₂O; (vi) Pd-C, cyclohexene.



Scheme 17. Synthesis of (D)- and (L)-3-hydroxy-4-hydroxymethyl cyclohexenyl nucleosides, part 2. (i) Bz₂O, DMAP; (ii) TBAF; (iii) (a) MsCl, Et₃N, (b) TBAF; (iv) MnO₂; (v) NaBH₄, CeCl₃·7H₂O; (vi) (a) PPh₃, DEAD, adenine or 2-amino-6-chloropurine, (b) TFA/H₂O.

formations mimic the two ³T₂ (north) and ³T₃ (south) conformations of furanose.

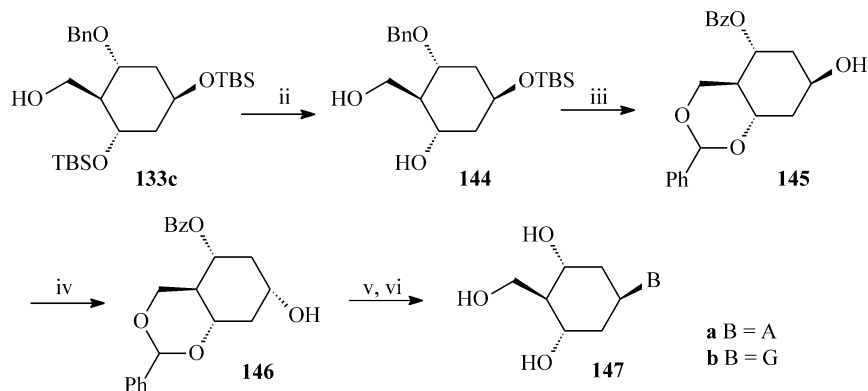
(*R*)-Carvone was converted into 2-methylenecyclohexanol intermediates **132a–c** in good yields via a sequence of *transformations*

(Scheme 16). Hydroboration with 9-BBN in THF gave the *trans*-hydroxymethyl substituted isomers **133a–c** as the major product in 70–78% yield.³¹

For the synthesis of D-3-hydroxy-4-hydroxymethyl nucleosides D-**138**, the primary hydroxyl group of **133a** was selectively protected and the remaining secondary alcohol was mesylated. Removal of the Bn protecting group of **134** and oxidation of the generated alcohol **135** with MnO₂ gave directly the enone **136**, which was reduced to the α -allylic alcohol **137** as the sole isomer. The purine bases were introduced using a Mitsunobu reaction and final deprotection gave the D-3-hydroxy-4-hydroxymethyl cyclohexenyl adenine and guanine derivatives D-**138a** and D-**138b**. Hydrogenation of the adenine D-**138a** gave D-3-hydroxy-4-hydroxymethyl cyclohexanyl adenine D-**47a**.

(L)-3-Hydroxy-4-hydroxymethyl cyclohexenyl adenine and its guanine counterpart L-**138** were synthesized in a similar way (Scheme 17).³² Diol **133b** was protected as its dibenzoate **139** and the equatorial TBDMS was selectively removed. Alcohol **140** was mesylated and the remaining TBDMS was cleaved. Following the same procedure as for the D-cyclohexenyl nucleosides the allylic alcohol **143** was generated, which was coupled with a purine base moiety using a Mitsunobu reaction, giving the L-cyclohexenyl guanine nucleosides L-**138**.

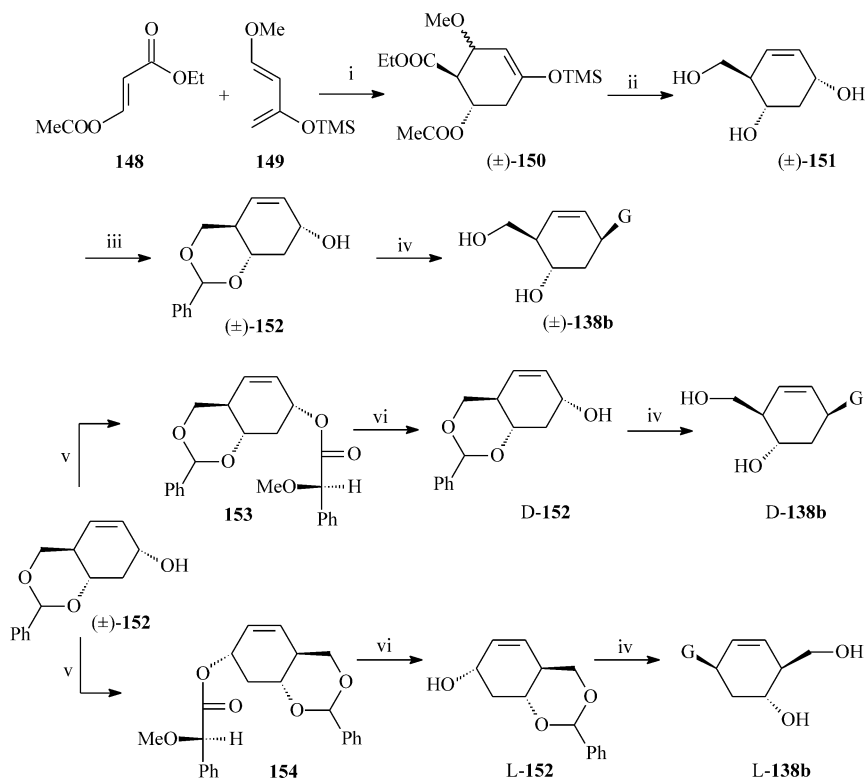
The same synthetic approach gave access to 3,5-dihydroxy-4-hydroxymethyl cyclohexanyl adenine and guanine **147** (Scheme 18).³³ The



Scheme 18. Synthesis of (D)- and (L)-3-hydroxy-4-hydroxymethyl cyclohexenyl nucleosides, part 3. (i) TBAF (1 eq); (ii) (a) PhCH(OMe)₂, PTSA, (b) TBAF; (iii) (a) PPh₃, DEAD, BzOH, (b) K₂CO₃, MeOH; (iv) (a) PPh₃, DEAD, dioxane, adenine, or 2-amino-6-chloropurine, (b) for adenine, 80% HOAc; for guanine, TFA/H₂O (3:1); (vi) Pd(OH)₂-C, cyclohexene.

equatorial TBDMS group of intermediate **133c** was cleaved and diol **144** was protected as benzylidene acetal **145**. Then the axial TBDMS group was removed and the configuration of the free hydroxyl group was inverted using a Mitsunobu reaction. Introduction of the purine moieties was carried out by a second Mitsunobu reaction with the α -alcohol **146**. Stepwise deprotection afforded the triol cyclohexanyl nucleosides **147**.

Recently, a straightforward synthetic approach towards the synthesis of 3-hydroxy-4-hydroxymethyl cyclohexenyl nucleosides was developed (Scheme 19).³⁴ Diels–Alder reaction of enone **148** and diene **149** generated the cyclohexene **150**, which was reduced with excess LiAlH_4 to the triol **151**. After protection as the benzylidene acetal **152**, the



Scheme 19. Synthesis of (D)- and (L)-3-hydroxy-4-hydroxymethyl cyclohexenyl nucleosides, part 4. (i) Hydroquinone, 180°C ; (ii) LiAlH_4 ; (iii) $\text{PhCH}(\text{OMe})_2$, PTSA; (iv) (a) PPh_3 , DEAD, 2-amino-6-chloropurine, (b) $\text{TFA}/\text{H}_2\text{O}$ (3:1); (v) (*R*)-(-)-methylmandelic acid, DCC, DMAP; (vi) NaOH , MeOH , H_2O .

guanine base was introduced under Mitsunobu reaction conditions to give racemic **138b**. Resolution of (\pm)-**152** via its chiral ester gave rise to both D- and L-**152**, from which D-**138b** and L-**138b** were prepared.

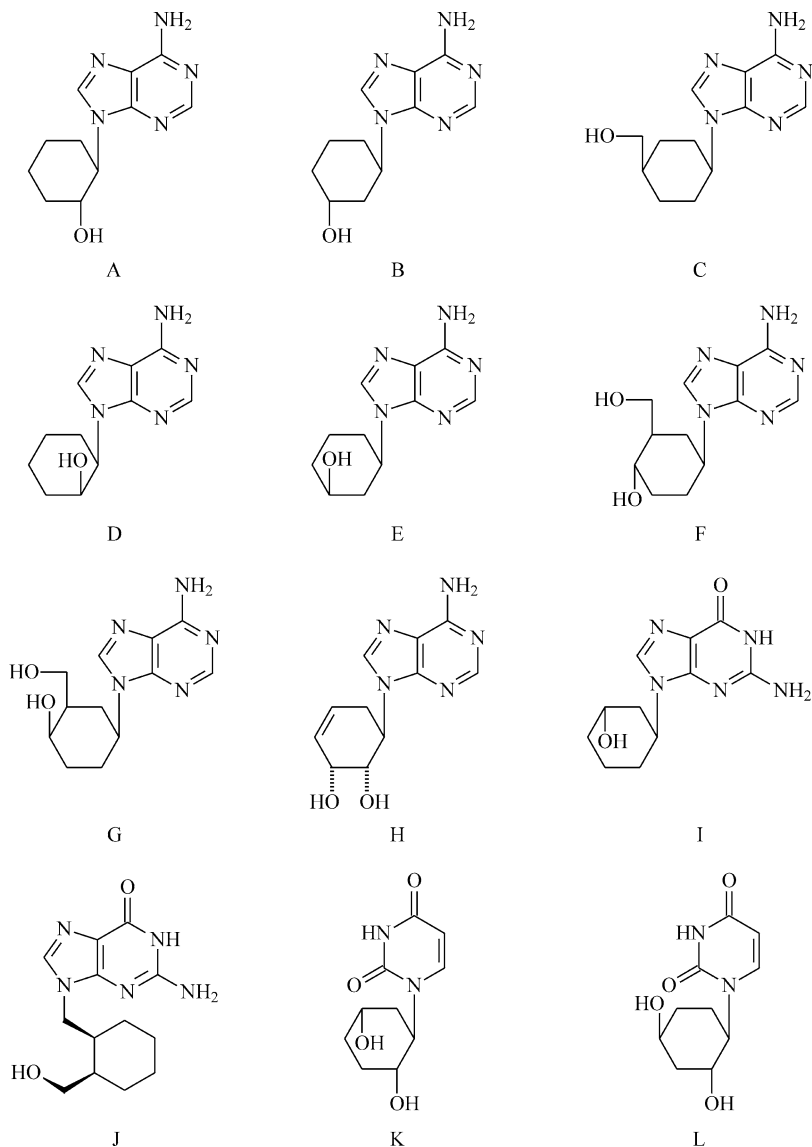
C. Cyclohexanyl and Cyclohexenyl Nucleoside Phosphonates

A limited number cyclohexanyl and cyclohexenyl nucleoside phosphonates as prodrug were reported. Rosenberg's group synthesized racemic *trans*- and *cis*-2-phosphonomethoxyethyl cyclohexanyl nucleosides **155** and **158** (Scheme 20).^{35,36} They were prepared by nucleophilic reaction of *trans*- or *cis*-2-hydroxycyclohexanyl nucleosides with diisopropyl tosyloxymethanephosphonate in the presence of NaH. The *cis*-2-hydroxycyclohexenyl nucleosides with purine bases were obtained by Mitsunobu reaction of the protected nucleobases with *trans*-2-benzyloxycyclohexanol **156**, whereas pyrimidine-containing nucleosides were obtained by configurational inversion at C-2 of the corresponding *trans*-2-hydroxy-cyclohexanyl nucleosides **7** via ring opening of their anhydro derivatives **159**.

The phosphonate derivatives of *cis*-4-hydroxy cyclohexenyl and cyclohexanyl nucleosides **163** and **164** were synthesized by Herdewijn's group. 1,4-*Trans*-cyclohexenediol **161** was monoprotected and the phosphonomethyl moiety was introduced, followed by deprotection to release the OH group. The heterocyclic base moieties were incorporated via a Mitsunobu reaction with inversion of configuration at the OH position. The diisopropylester group of the phosphonates **162** was cleaved with trimethylsilyl bromide to give the cyclohexenyl nucleoside phosphonates **163**.³⁷ The cyclohexanyl phosphonate derivatives **164** were obtained via hydrogenation of the double bond and deprotection of the phosphonate ester group.³⁸ All synthesized phosphonates were inactive against herpes simplex virus (HSV) and human immunodeficiency virus (HIV).

IV. BIOLOGICAL ACTIVITIES AND COMPUTATIONAL MODELLING

Carbocyclic six-membered nucleic acids have never been extensively and systematically screened for their antiviral activity. Some of the compounds have been tested against HSV and/or HIV, but most of these carbocyclic nucleosides were found to be inactive.^{4,15,19,25,26,29,33,38} Compounds in Table 1 (**A** \rightarrow **G**) were described as adenosine deaminase inhibitors^{1-3,11} and compound **H** as inhibitor of bovine AdoHcy hydrolase (Scheme 21).²⁸



Scheme 21. Carbocyclic six-membered nucleosides demonstrating biological activity.

clovir in HSV-1 infected cells, which was attributed to its ability to inhibit HSV-TK.⁶ More profound studies have been carried out with compound **J**.⁸⁻¹⁰ This compound is a potent and selective HSV-1 TK in-

hibitor (IC_{50} : 0.07 μ M). It delayed the reactivation of latent virus from explanted mouse ganglia but exacerbated the primary HSV-1 infection in mice. It is not a substrate for HSV-1 TK and is ineffective against virus replication in cell culture. Compound **K** is described as a weak inhibitor of cytomegalovirus (CMV) (IC_{50} : 1 μ M) and HSV-2 (EC_{50} : 1 μ M), while its regio isomer (**L**) is a weak anti-HSV (type 1 and type 2) compound (EC_{50} : 1 μ M).⁵

The most potent six-membered carbocyclic nucleoside is cyclohexenyl-G³² (D-138-B). Both the D- and L-analogues show antiviral activity, although the difference in antiviral activity is larger than originally reported.³² D-Cyclohexenyl-G is active against HSV-1 at 0.005 μ g/mL, while the L-congener is active at 16 μ g/mL. D-Cyclohexenyl-G³² is also active against HSV-2 (0.05 μ g/mL), varicella-zoster virus (VZV) (0.5 μ g/mL) and CMV (0.5 μ g/mL), while showing no cell toxicity at 200 μ g/mL. Molecular modelling revealed that, both D- and L-cyclohexenyl-G can be docked in the active site of HSV-1 thymidine kinase and that the same amino acids are involved in binding both enantiomers. The difference in interaction energy, however, is about 20 kcal/mol, which might partially explain the differential antiviral activity of both compounds.

The CeNA D and L enantiomers with a guanine base were docked into the active site of HSV-1 thymidine kinase (TK).³² Their energies were optimized using the amber software package. The orientation of both enantiomers in the active site is the same: both molecules have a pseudo-equatorial conformation when bound to the TK active site. Both molecules adopt the syn conformation, the torsion angle χ [C6'-C1'-N9-C4] is 18.8 degrees in **4** and -88.5 for molecule **6**. The guanine bases are in the same position stabilized by a network of hydrogen bonds superimposed on a stacking interaction with Tyr-172. Tyr-101 OH group is out of reach of the sugar 3'-OH in contrast to the complexes of TK with acyclic guanosine analogs like ganciclovir and penciclovir.³⁹ Instead, the 3'-hydroxyl group is only H-bonding to the Glu-225 OE2 atom. The D- and L-cyclohexenyl sugars occupy the same region with the C5' and C3' pointing in the same direction. However, due to the difference in configuration, the 3'-OH group of the D-isomer can get closer to the Glu-225 side chain resulting in a much stronger interaction than in the L-enantiomer (distance 4.03'... Glu-225.OE2 2.42 Å, 6.03'... Glu-225.OE2 3.58 Å). This is reflected in an amber interaction energy of -99.1 kcal/mol (van der Waals -22.6 , electrostatic -76.5 kcal/mol) for the D-cyclohexenyl-G compared to -79.0 kcal/mol (van der Waals -29.1 , electrostatic -49.9 kcal/mol) for L-cyclohexen-

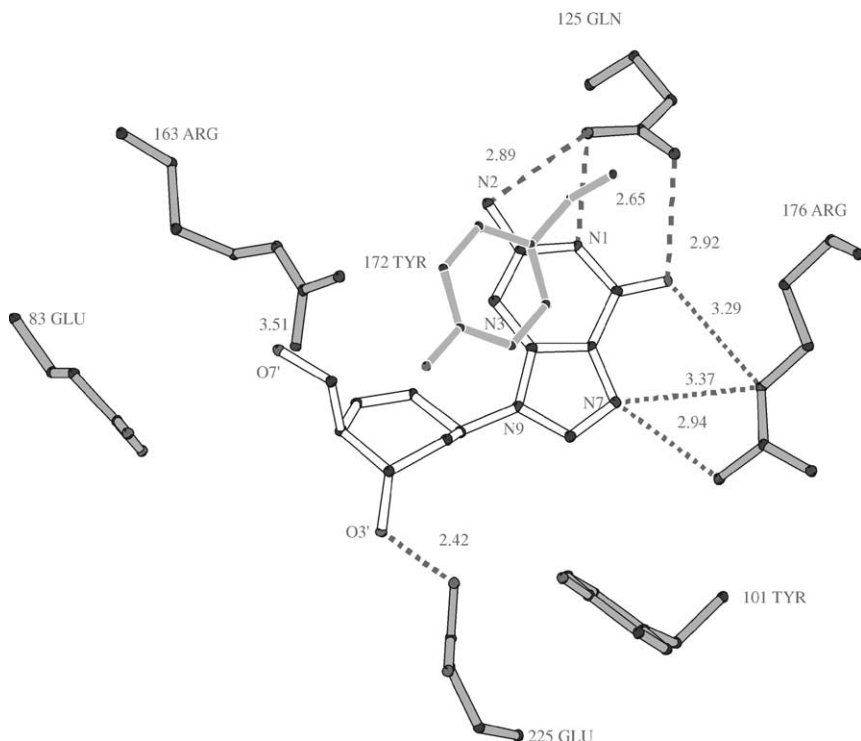


Figure 1. Docked D-enantiomer of CeNA in the active site of HSV-1 thymidine kinase. Hydrogen bond distances are indicated in Å.

yl-G. Figure 1 shows the docked D-isomer in the active site of the TK enzyme.

V. CONCLUSIONS

The development of six-membered carbocyclic nucleosides is still in its infancy. The number of compounds synthesized as well as the available synthetic schemes is limited. Nevertheless, several compounds have been identified as enzyme inhibitors and/or as antiviral agents. High antiviral activity was found with compounds having a rather flexible cyclohexene ring with D-cyclohexenyl G as most important example. Its potential use as an antiviral drug has to await further *in vivo* investigations in animal models. From the drug discovery point of view, there is plenty of opportunity for new synthetic projects and for new findings in both fundamental and applied biology.

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*cyclo*SAL-PRONUCLEOTIDES—DESIGN OF THE CONCEPT, CHEMISTRY, AND ANTIVIRAL ACTIVITY

Chris Meier

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I. INTRODUCTION

Nucleoside analogues, e.g., 2',3'-dideoxy-2',3'-didehydrothymidine **1** (d4T), 3'-azido-2',3'-dideoxythymidine **2** (AZT) or 5-fluoro-2'-deoxyuridine **3** (5-FdU), are structurally different as compared to the corresponding natural DNA or RNA nucleosides with regard to modification of the glycon as well as the aglycon residue. Due to this modified structure, these compounds are widely used as antiviral or antitumor drugs in chemotherapy (Figure 1).¹ Since the discovery of AZT **2** as the first nucleoside drug for the treatment of AIDS,² considerable efforts have been made to develop new nucleoside analogues that would be more active, less toxic inhibitors of the HIV-1 reverse transcriptase (RT).³ Today, synthetic nucleoside mimetics represent a highly valuable source of antiviral agents that contribute significantly to the arsenal of agents for the treatment of diseases ranging from cancer, herpes, and hepatitis to AIDS. Recently, nucleosides having the unnatural L-configuration (e.g., β -L-2',3'-dideoxy-3'-thiacytidine (3TC **4**)) have been found to possess interesting biological activity.⁴ The nucleoside analogues inhibit the HIV-1 RT or viral DNA polymerases by acting as competitive inhibitors and/or as DNA chain terminators. To act as DNA chain termination agents or polymerase inhibitors, intracellular conversion of the

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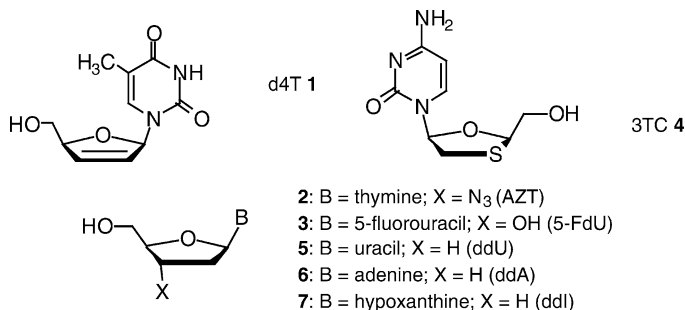


Figure 1. Examples of nucleosides used as antiviral and antitumor drugs.

nucleoside analogues into their 5'-mono-, 5'-di-, or 5'-triphosphates, after cell penetration, is a prerequisite.⁵ The enormous disparity in anti-HIV activity that is evident for a large number of dideoxynucleoside analogues contrasts with their apparent structural similarity. However, due to the structural differences as compared to natural nucleosides the efficient metabolization to the corresponding dideoxynucleoside triphosphates is often the major hurdle and, consequently, the therapeutic efficacy is sometimes limited.⁶ For example, in the case of the anti-HIV active dideoxynucleoside analogue d4T **1** (Stavudine, Zerit®)⁷ the first phosphorylation to d4T 5'-monophosphate catalyzed by thymidine kinase (TK) is the rate-limiting step in human cells.⁸ More striking, however, is the case of 2', 3'-dideoxyuridine triphosphate (ddUTP), which is one of the most powerful and selective inhibitors of HIV reverse transcriptase ($K_i = 0.05 \mu\text{M}$), while the parent nucleoside 2', 3'-dideoxyuridine **5** (ddU) is virtually ineffective in blocking HIV infection in cultured cells.^{6b} Biochemical and pharmacological studies in three different human T cell lines (CEM, ATH8, and Molt-4) showed that ddU **5** itself was not anabolized to the 5'-monophosphate, most apparently because it was a poor substrate for cellular nucleoside kinases because of the considerable substrate specificity of these enzymes.⁹ In contrast, in a few cases the limited efficacy is also due to a catabolic enzymatic reaction, e.g., 2', 3'-dideoxyadenosine **6** (ddA) is rapidly deaminated intracellularly to ddl **7** by adenosine deaminase (ADA) (further details to this metabolism will be given later). Finally, the resistance of the human immunodeficiency virus to the clinically used antiviral dideoxynucleoside AZT **2** (Zidovudine, Retrovir®)¹⁰ is on one hand directly related to multiple point mutations within the HIV-1 reverse transcriptase. On the other hand, it may be also due to lower susceptibility of the target cells related with a decreased activity or in-

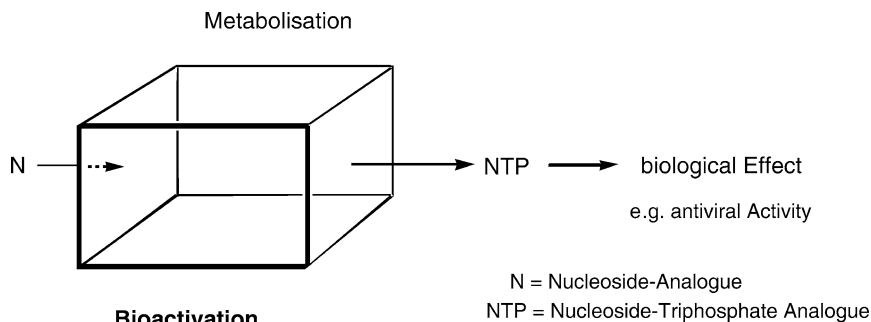


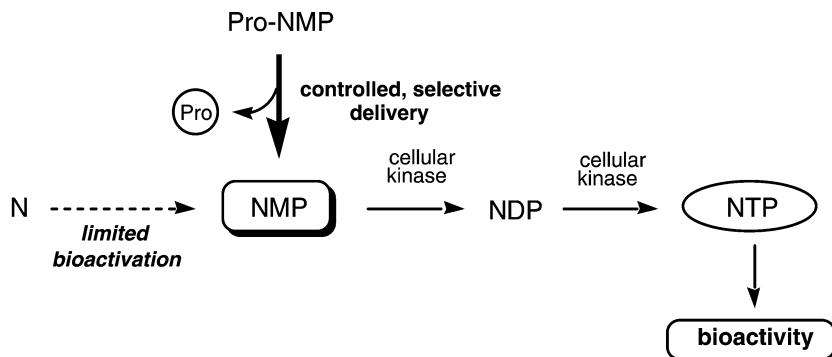
Figure 2. “Black-box-metabolism” of potential biologically active nucleoside analogues.

ability of the enzyme thymidine kinase to phosphorylate AZT **2** to the dideoxynucleoside monophosphate AZTMP.¹⁰

Despite of the above given examples, it should be mentioned that for the great majority of nucleoside analogues the intracellular metabolism has not been studied in detail. Often these compounds are only tested as their nucleoside forms and—if biologically inactive—they are not further pursued. They have rarely been studied as their triphosphates. A metabolic block within the biosynthesis of the nucleoside triphosphate may abolish all potential antiviral activity. To the author's opinion it may be a big fault to trust only in the “black-box-metabolism” of the parent nucleosides (Figure 2). A few examples that support this opinion will be given in this overview.

In contrast, a detailed knowledge of the metabolism, or lack thereof, during the multi-step phosphorylation may offer the chance to develop compounds with improved biological potential. Therefore, in the case of the nucleosides d4T, ddA, and AZT, the direct administration of their nucleotides should bypass the limiting step(s) in their metabolism and this should have advantages for the biological activity. Unfortunately, because of the high polarity of the nucleotides, these compounds are not able to easily penetrate cellular membranes or the blood–brain barrier. However, the phosphate moiety offers a suitable site to attach degradable lipophilic carrier residues. As a result, one effort to improve the therapeutic potential of nucleoside analogues is the delivery of the corresponding nucleotides from neutral, membrane-permeable prodrugs (*pronucleotide approach*; Figure 3).¹¹

A lipophilic phosphate triester may penetrate into the target cell where first partial and at the end complete hydrolysis releases the nucleotide.



N: Dideoxynucleoside analogue

Figure 3. General principle of the pronucleotide approach of biologically active dideoxynucleotide analogues.

A suitable nucleotide prodrug has to fulfill two requirements: (i) it has to be sufficiently lipophilic for passive diffusion of the membrane and blood–brain barrier; (ii) furthermore, it should be able to deliver the nucleotide hydrolytically or enzymatically leaving a non-toxic masking group.¹²

In principle, two different concepts for prodrug design are known: bipartate and tripartate prodrugs. In the former concept, the drug is masked with a one-component group. In this form the drug is biologically inactive. After a simple cleavage of the mask, the active drug is liberated (Figure 4). In the latter concept, the drug is modified with a two-component masking group. Again, the drug is biologically inactive in this bound form. The mechanism of liberation involves a first chemical or enzymatic reaction under cleavage of part I of the masking moiety. The drug is still inactive but the effect of this first reaction is an activation of the remaining masking group II with the consequence of a spontaneous successive cleaving reaction releasing the now bioactive drug (cascade-mechanism; Figure 4).¹³

In the case of a nucleotide prodrug one should take into account that under physiological conditions two negatively charged phosphate oxygen's have to be masked in order to obtain a neutral, lipophilic phosphate ester. Consequently, not only one masking group is necessary but two. So, the efficient intracellular delivery of nucleotides from a prodrug requires the existence of a specific delivery mechanism or different rates of conversion of the prodrug to the drug intracellularly *versus* extracellularly. One comment with respect to toxic side events should be given. Neutral phosphorus derivatives with a good leaving group

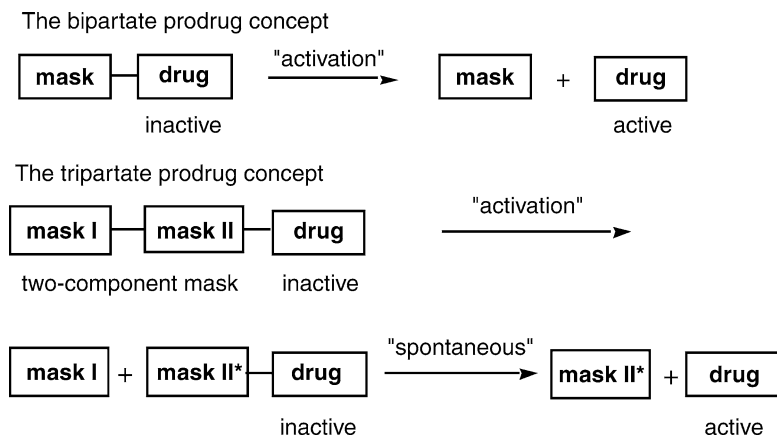


Figure 4. General principle of prodrugs bearing one-component or two-component masking groups.

attached to the phosphorus are known to be toxic suicide inhibitors of acetylcholinesterase.¹⁴ The anti-acetylcholinesterase activity of phosphorus derivatives is an inverse function of the pK_a of the leaving group on the phosphorus atom and parallels the rate of spontaneous hydrolysis by P–O bond cleavage.¹⁵ In order to circumvent the possible problem of anti-acetylcholinesterase activity, neutral phosphate ester prodrugs should be designed to undergo heterolytic cleavage of the C–O bond rather than the P–O bond of the ester.

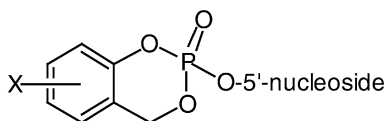
Many strategies have been developed to achieve this goal. In general, uncharged nucleotide triesters have been used as membrane-permeable nucleotide precursors.¹¹ The major differences of these approaches are the delivery mechanisms to the nucleotides. First attempts have been made with simple dialkyl phosphotriesters. These compounds generally belong to the class of bipartate prodrug systems. After a first, sometimes selective hydrolysis of the phosphate triester *via* a nucleophilic reaction at the phosphorus center, the resulting phosphate diester is often extremely stable against a further chemical hydrolysis due to the charge at the phosphate that prevents a second nucleophilic reaction.¹⁶ Even if the subsequent hydrolysis is possible, a selective delivery of the nucleotide is not always possible due to the pseudorotation phenomenon during phosphate ester hydrolysis.¹⁷ As a consequence, almost all approaches based on chemical hydrolysis reported so far, except for the *cycloSal* approach, were unable to deliver the nucleotide selectively. For this reason, the newer pronucleotide ap-

proaches are based on the concept of a tripartate prodrug system¹³ and are based on the general idea of a selective chemical or enzymatic reaction within the masking group which leads to a second, spontaneous successive reaction yielding the charged phosphate ester. These approaches utilize and exploit the differences in enzyme activity and pH value. The concepts working with enzymatic trigger processes are the bis(POM)-,¹⁸ bis(POC)-,¹⁹ bis(DTE)-,²⁰ bis(SATE)-,²¹ bis(SGTE)-,²² bis(AB)-,²³ the aryloxyphosphoramidate (APA),²⁴ the phosphoramidate monoester,²⁵ and a modified SATE-concept.²⁶ All of the aforementioned enzyme-driven concepts have demonstrated the successful intracellular delivery of free nucleotides from the lipophilic pronucleotide. In this review, the so-called *cycloSal* approach developed in our laboratory will be described in detail.

II. *cycloSal*-NUCLEOTIDES—DESIGN OF THE CONCEPT

We have developed the first pronucleotide system that successfully delivers nucleotides by simple chemical activation involving a highly selective coupled two-step mechanism (cascade mechanism): *cycloSal*-saligenyl-nucleoside monophosphates (*cycloSal*-NMP,¹ Figure 5).^{27,28} The concept was successfully applied to different antivirally active nucleoside analogues, as will be shown later.

In contrast to all approaches mentioned previously, our aim was the development of a highly selective delivery mechanism that is



cycloSal-nucleoside monophosphate (*cycloSal*-NMP)

nucleosides: d4T, AZT, FdU, ddA, d4A

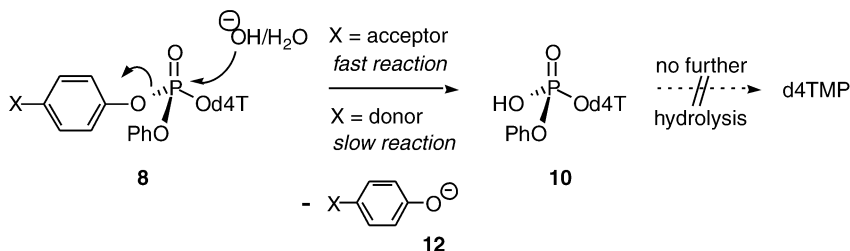
F- α -ddA, F- β -ddA, ACV, PCV, BVDU

X: 5-, 3- and 3,5-alkyl, 5- and 3-methoxy,
5-nitro and 5-chloro

Figure 5. General structure of the *cycloSal*-phosphate triesters.

¹The correct systematic name would be 2-Nucleos-5'-O-yl-4H-1,3,2-benzodioxaphosphorinin-2-oxides. As a simplification the abbreviation *cycloSal*-NMP has been introduced by us and will also be used in this review.

a) hydrolysis of bis-phenyl phosphate diesters



b) hydrolysis of bis-benzyl phosphate diesters

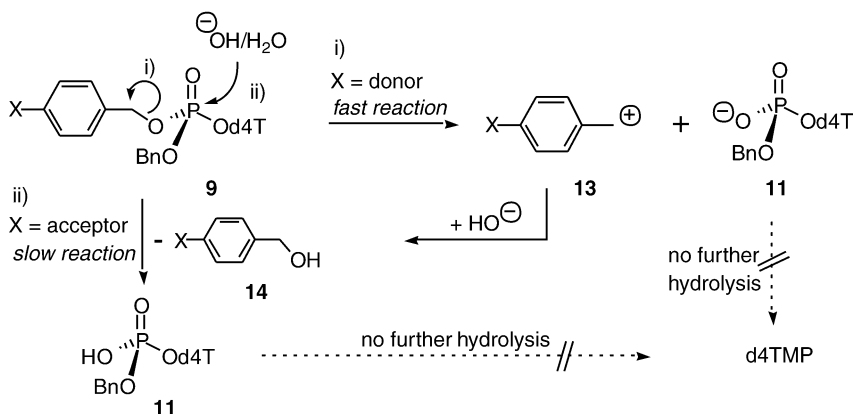


Figure 6. Hydrolysis pathways of bis-phenyl- (**8**) and bis-benzyl phosphate triesters **9**.

based on a purely pH-dependent chemical reaction. However, as mentioned above, the chemically driven release of a monophosphate from a lipophilic precursor is not as easy as it seems. Earlier studies in our and other laboratories showed that bis-phenyl- (**8**)²⁹ as well as bis-benzyl phosphate triesters (**9**)³⁰ showed a selective hydrolysis to yield the phenyl- (**10**) and the benzyl phosphate diesters (**11**), respectively, but there was not further hydrolysis observed to give the nucleoside monophosphates (Figure 6).¹⁶ As a consequence, all these compounds proved to be completely antivirally inactive in *in vitro* cell assays against HIV. Nevertheless, the studies using bis-phenyl phosphate triesters **8** showed the expected dependence of the hydrolytic stability of the substitution pattern in the aromatic ring: the more electron-withdrawing the substituent, the more labile the phosphate triesters was.²⁹ The situation was completely inverted when we stud-

ied bis-benzyl phosphate triesters **9**: here, the more electron-donating the substituent, the more labile was the compound.^{30–32} Interestingly, the mechanisms of hydrolysis were entirely different.

An initial attack of an external nucleophile at the phosphorus center of the bis-phenyl phosphate triester led to the cleavage of the P–O_{phenyl} bond to give the phosphate diester **10** and a phenolate anion **12**. In contrast, a spontaneous C_{benzyl}–O bond cleavage led to the formation of the benzyl phosphate diester **11** and a benzyl cation **13** starting from the bis-benzyl phosphate triester **9**. The cation **13** was subsequently quenched by water to give a benzyl alcohol **14**. Moreover, the half-life of the bis-benzyl phosphate triesters **9** are extremely low ($t_{1/2} < 15$ min) which makes a drug delivery system based on this type of phosphate triester not useful.^{31,32} Nevertheless, both reactions are quite selective but further hydrolysis to yield the nucleotide could not be detected. Even enzymatic degradation of the intermediate diesters proved to be problematic.³³ The reason is the formed negative charge at the phosphorus atom which prevents a second nucleophilic attack in the former case and at the same time decreases the leaving group properties of the 5'-nucleoside phosphate fragment dramatically in the latter case. Additionally, it is clear that for both types of phosphate triesters two independent reaction steps should take place in order to convert the triester into the nucleotide itself. Obviously, the problematic step is the cleavage of the intermediately formed phosphate diester from the original phosphate triester.

Our idea was to combine the hydrolysis properties of the described phenyl or benzyl phosphate triesters. The aim was to develop a degradation mechanism that would allow—although it is still a two-step-mechanism—a selective release of the nucleotide and the masking group by a controlled, chemically induced coupled process that takes place within the masking unit (tandem or cascade mechanism). If such a concept works, the coupling of the two ester hydrolysis steps would circumvent the limitations of the chemical¹⁶ and sometimes even the enzymatic hydrolysis³³ of the intermediate phosphate diester intermediates. Therefore, our concept was to introduce three different ester bonds to the phosphate group: a phenyl-, a benzyl-, and an alkyl phosphate ester. Only the introduction of these three ester bonds would allow to discriminate sufficiently between the different phosphate ester bonds with the result of a design of a new chemically cleavable tripartate prodrug system.¹³ The alkyl ester represents the most stable ester bond within the triester molecule. Therefore, only the phenyl and the benzyl ester play a role in the delivery mechanism. Furthermore, because of the integration of a phenyl and a benzyl ester in

the same masking unit we may achieve a cleavage process that—after a first reaction involving the phosphorus atom—only takes place within the masking group. Hence, no second hydrolytic reaction at the phosphorus atom is needed. This would completely exclude a possible pseudorotation process that may lead to the liberation of the nucleoside instead of the nucleotide. The rationale of our new *cycloSal*-prodrug concept is based on the described difference in stability of the phenyl- and the benzyl phosphate ester and was tested using d4T **1** as the nucleoside (*cycloSal*-d4TMP **15**).^{34,35} The phenyl ester bond should be the most labile one because the negative charge could be delocalized in the aromatic ring leading to the 2-hydroxybenzylphosphate diester **16** (step a, Figure 7). The alternative cleavage of the benzyl ester to yield the 2-hydroxymethylphenylphosphate diester **17** is unfavorable (step c, Figure 7), because the phosphate residue (electron-withdrawing group) in the ortho-position of the benzyl ester stabilizes this bond. Hence, in the initial step, the phenyl ester is cleaved selectively so that the ortho-substituent to the benzyl group is changed from an electron-withdrawing group (phosphate) to an electron-donating (hydroxyl) group. As a result, this “Umpolung” of the 2-substituent intrinsically activates the remaining masking group and this induces a spontaneous cleavage of diester **16** to yield the nucleotide d4TMP and the diol mask **18** (tandem reaction; step b, Figure 7).

The main difference to the aforementioned chemically stable and enzymatically activated pronucleotide concepts is that our concept requires *one* activation step to deliver the nucleotide only. Moreover, in contrast to all other pronucleotide systems reported so far, following our concept only *one* masking molecule per nucleotide has to be used because the *cycloSal* group functions as a bifunctional masking group. Other strategies use up to four masking molecules per nucleotide (mask: nucleotide ratio of 4:1).³⁵

It should be added that following our concept neither the potentially toxic compounds formaldehyde, or episulfide^{36,37} nor pivalic acid³⁸ is formed. However, as in the mentioned bis(AB) approach,^{23a-d} we may generate a 2-quinonemethide intermediate **19**.³⁹ But, in contrast to the bis(AB) concept, we should not witness formation of this species in close vicinity of an enzyme active site because no enzyme is required to activate the *cycloSal* compounds. So, the 2-quinonemethide **19** should be rapidly quenched by water to give the salicylalcohols **18**. For this reason, in addition to the *cycloSal* phosphate triesters, the differently substituted salicylalcohols **18** were also tested for their antiviral or toxic potency. EC₅₀ values as well as CC₅₀ values > 250 μM were found in human lymphocytic CEM cells.³⁵ Apparently, the salicylalco-

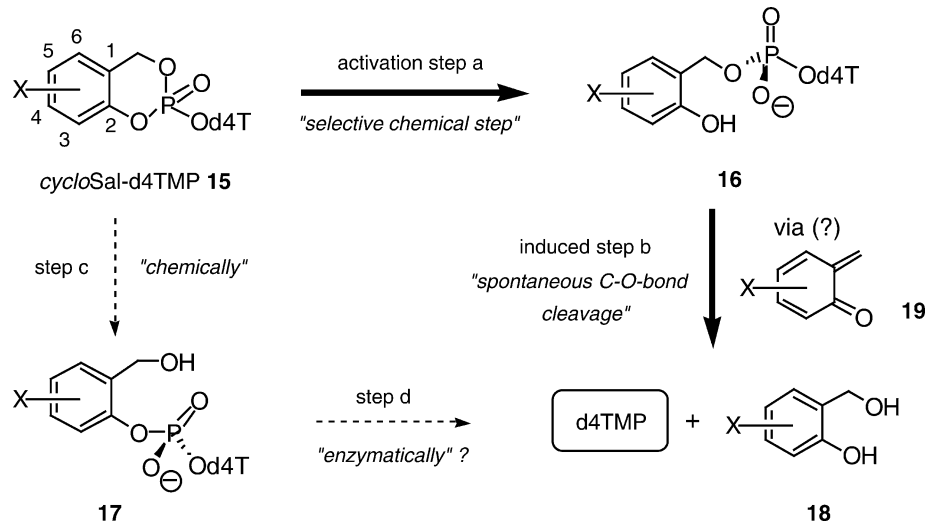


Figure 7. Two possible hydrolysis pathways of *cycloSal*-d₄TMP triesters **15**.

holds **18** are neither antivirally active nor toxic. The same was found when FM3A cells were used: EC_{50} values $> 350 \mu\text{M}$ were detected.⁴⁰ Moreover, in mice doses up to 250 mg/kg of different diols did not cause any measurable toxic side effects.⁴¹ It should be added that salicylalcohol (saligenin) is used as part of the antirheumatic and analgetic drug Salicin (2-[hydroxymethyl]phenyl- β -D-glucopyranoside) that is available under the trade name Assalix.⁴² Salicin is enzymatically hydrolyzed to saligenin and glucose by β -glucosidase. Saligenin is slowly oxidized by cytochrome P450 to salicylic acid in the blood and in the liver.⁴³

III. CHEMISTRY

The synthesis of the *cyclo*Sal-pronucleotides has been done most successfully by using reactive phosphorus(III)-reagents (part a, Figure 8).^{34,35} Due to the fact that the nucleoside analogues are the most expensive part of the triester molecule, the nucleoside was introduced at the latest possible step in the reaction sequence. Therefore, salicylalcohol **18** was reacted with phosphorus trichloride to give the cyclic chlorophosphites **20**. These compounds could be easily purified by Kugelrohr-distillation. The phosphites may be stored at -20°C for at least one year. The cyclic phosphites **20** then were reacted either directly with the nucleoside analogue, e.g., d4T in the presence of diisopropylethylamine (DIPEA; Hünig's base) to yield the cyclic phosphite triesters **21** which were oxidized in a one-pot-reaction by addition of *t*-butylhydroperoxide (TBHP) or dimethyldioxirane. So, the phosphate triesters **15** were obtained in reasonable yields as diastereomeric mixtures. Alternatively, the cyclic chlorophosphite **20** was treated with two equivalents of diisopropyl amine in diethylether leading to the formation of the corresponding phosphoramidite **22**.⁴⁴ After filtration of the precipitated diisopropyl ammoniumchloride the solvent was distilled off and the crude product was pure enough to be used directly for the 5'-phosphitylation reaction. The coupling with the nucleoside analogue was carried out in acetonitrile in the presence of pyridinium chloride or 1H-tetrazole as coupling activator. Both activators led to comparable yields. Even better as coupling reagent was imidazolium triflate. Using this coupling activator yields of $> 90\%$ were obtained. The purification was done by water extraction of the salt and subsequent silica gel chromatography on a Chromatotron. The latter phosphoramidite methodology had dramatic advantages for a few nucleoside analogues: 3TC **4** for example yielded a mixture of 5'-O-phosphorylation and 5'-O,N⁴ diphosphorylation when the nucleoside was reacted with the

a) phosphorus (III) chemistry

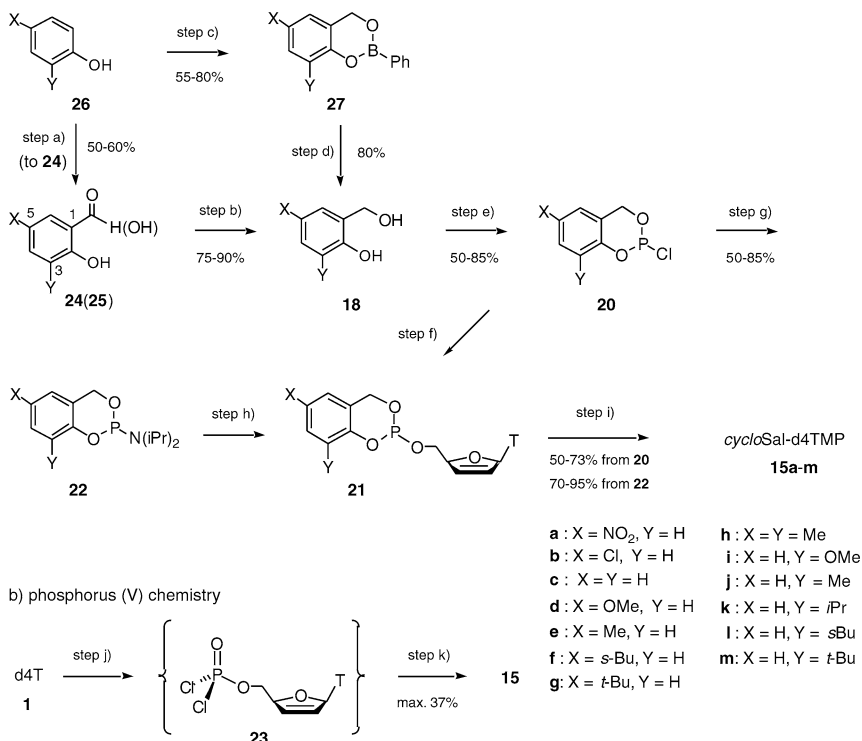


Figure 8. Synthetic pathways to the diastereomeric mixtures of the *cycloSal*-d4TMP triesters **15a–m**. Reaction conditions: (a) Casiraghi-Formylation;⁴⁵ (b) NaBH₄ for **24** (LiAlH₄ or NaBH₄/I₂ for **25**); (c) Nagata-hydroxyformylation;⁴⁷ (d) H₂O₂, MeOH; (e) PCl₃, pyridine, Et₂O, –10 °C, 2 h; (f) d4T **1**, DIPEA, CH₃CN, 0 °C, 20 min; (g) diisopropyl amine (2 eq), diethylether, 0 °C, 30 min; (h) pyridinium chloride, tetrazole, or imidazolium triflate, CH₃CN, 0 °C, 30 min; (i) TBHP or dimethyldioxirane, CH₃CN, rt, 30 min; (j) P(O)Cl₃, DIPEA, THF, 0 °C, 12 h; (k) salicylalcohol **18**, DIPEA, THF, 0 °C–rt, 12 h.

chlorophosphites **20**, while only the desired 5'-O-phosphate triester was obtained following the phosphoramidite protocol.⁴⁴ Another example is the acyclic purine nucleoside analogue acyclovir (ACV). The use of the chlorophosphites **20** gave varying yields of 25–50% of the corresponding phosphate triester. Using the phosphoramidite **22** again improved not only the reproducibility of the reaction but also the yield to ca. 80%.³⁰

Experiments using directly phosphorus(V) reagents—independently if first the nucleoside analogue was attached to P(O)Cl₃ (leading to

23) or the masking unit—led always to lower yields of the desired phosphate triesters (part b, Figure 8).³⁵ The salicylalcohols **18** used as the masking group were prepared from the corresponding salicylic acids **25** or -aldehydes **24** by standard reduction protocols. In a few cases the acids or the aldehyde, respectively, were not commercially available. Then we prepared the salicylalcohols from the corresponding phenol derivatives **26**. Selective ortho-formylation was possible by the Casiraghi procedure⁴⁵ (paraformaldehyde/tintetrachloride) or the Rieche formylation protocol.⁴⁶ However, the latter method cause severe problems when we scaled up the reaction to about 10 g amounts due to overalkylation of the aromatic ring. Both methods led first to the salicylalcohols that had to be reduced in a subsequent reaction. Another alternative for the synthesis of the salicylalcohols is the direct hydroxymethylation according to the Nagata procedure.⁴⁷ Phenols **26** were treated with paraformaldehyde in the presence of phenylboronic acid and a catalytic amount of propionic acid to give a cyclic phenylboronic diester **27** that was cleaved oxidatively to yield the salicylalcohol **18**, phenol, and boronic acid. The latter method has the enormous advantage that even ester functionalisation in the aromatic ring would be unaffected while in the case of the Casiragi formylation transesterification reactions were a great problem. A schematic summary of the preparation of the different *cycloSal*-d4TMP triesters **15** is shown in Figure 8.

IV. *cycloSAL*-D4TMP PRONUCLEOTIDES— THE PROOF-OF-PRINCIPLE

Various studies on the properties of the *cycloSal*-phosphate triesters were carried out. First, we conducted a NMR experiment of 5-nitro-*cycloSal*-d4TMP **15a** in DMSO- d_6 containing 10% water. This mixture has been used for an initial study concerning the selective degradation because the possible or expected hydrolysis products as well as the benzyl phosphate diester intermediate may be detected by multinuclear ^{31}P -, ^{13}C -, and ^1H -NMR spectroscopy.^{34a} As shown in Figure 9, at the beginning of the hydrolysis the two resonance signals of the diastereomeric phosphate triesters were detected as the major products. However, there is already a signal visible at 0.2 ppm (section A). This peak increases with time while the two resonance signals of the triesters decreased (section B). The inset in section C shows a proton coupled ^{31}P -NMR of the peak at 0.2 ppm. It is obvious that the singulett splits into a quintett, which indicates that four protons are in

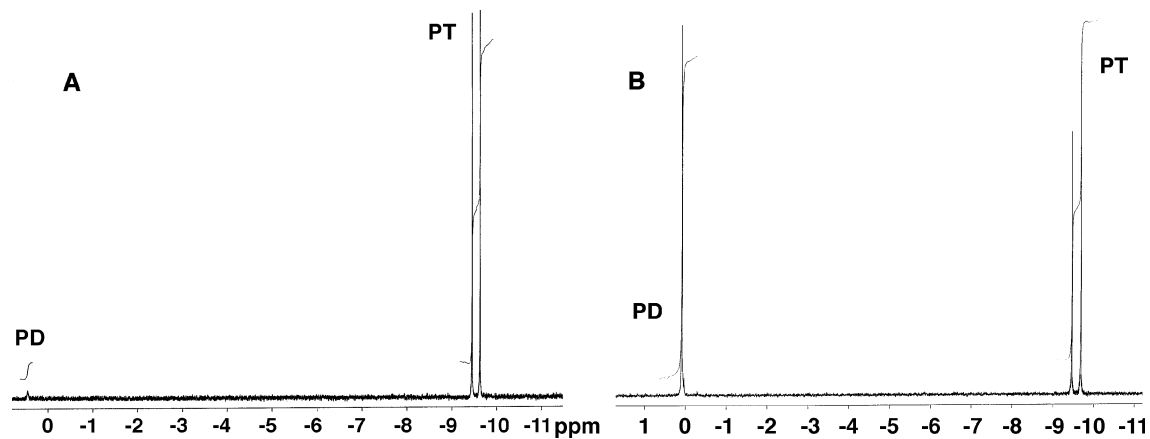


Figure 9. ^{31}P -NMR spectra of the hydrolysis of 5-nitro-cycloSal-d4TMP **15a** in DMSO/water. PT: phosphate triester **15a**; PD: phosphate diester **16a**; PM: phosphate monoester d4TMP.

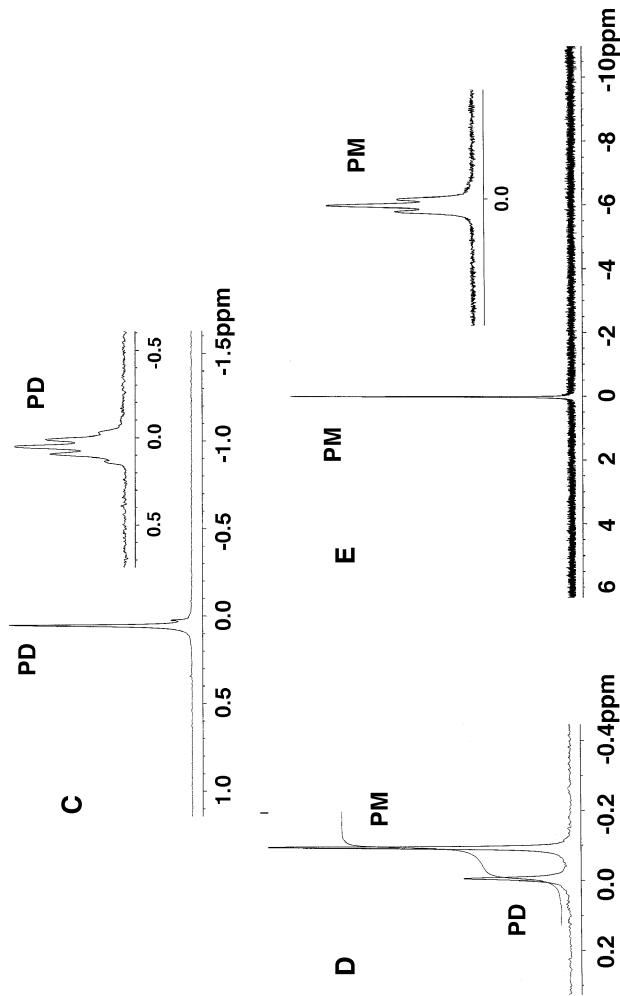


Figure 9. — (Continued).

the vicinity of and therefore coupling with the phosphorus atom. These four protons consist of two protons at the 5'-position of d4T and two protons at the benzyl position of the masking unit. As a consequence, the expected bond cleavage of the phenol ester bond took place leading to the benzyl phosphate diester intermediate **16** (Figure 7). Another spectroscopic evidence of the benzyl diester **16** is the upfield shift of the ipso-carbon atom with respect to the phenol-oxygen atom of about 12 ppm. At the same time this proves also the "Umpolung" of the substituent ortho to the benzyl ester bond from an electron-withdrawing group into an electron-donating group. It was interesting to observe that the resonance signal of diester intermediate decreased again and a new signal at -0.1 ppm appeared in the spectrum (Figure 9, section D). At the end of the reaction the signal of the only phosphorus containing product split into a triplet which is a good indication for the nucleotide d4TMP (coupling to the C5'-protons) (section E). In the ^1H -NMR spectrum we detected the ^1H -resonance signals of d4TMP as well as 5-nitrosalicylalcohol in a 1:1 ratio, which is also in accordance to the peaks in the ^{13}C -NMR spectrum. This experiment thus confirmed the designed hydrolysis pathway.

Chemical hydrolysis studies of the *cycloSal*-d4TMP triesters **15** proved the exclusive degradation to the nucleotide d4TMP as well as the formation of the salicylalcohols **18**.^{34,35} These experiments were done in different aqueous buffers and in RPMI-1640 culture medium with 10% heat-inactivated fetal calf serum. Furthermore, a clear correlation of the electronic properties introduced by the salicylalcohol substituents and the hydrolysis half-lives of the phosphate triesters was observed.³⁵ This is summarized in Table 1. It is worth mentioning that we observed a change in the rate-limiting step of the degradation of the *cycloSal*-d4TMPs. While in the case of electron-withdrawing substituents in the aromatic ring (**15a,b,i**) the cleavage to the phosphate diester **16** is a fast reaction and the second cleavage is rate-limiting, the situation is inverted in the case of electron-donating substituents. This could be concluded from the HPLC-hydrolysis studies: in the chromatograms of the hydrolyses of the former compounds the intermediate phosphate diester **16** could be detected while this was not possible for the latter compounds. Moreover, the expected pH-dependence of the nucleotide delivery typical for the chemical hydrolysis was observed at pH 6.8, 7.3, and 8.9 (Table 1).³⁵

No evidence of an enzymatic degradation in the RPMI-1640 medium containing 10% FCS has been observed so far. Studies in CEM-cell extracts showed that the hydrolysis half-lives only slightly decreased as compared to the buffer hydrolysis.⁴⁸ This confirms the initial idea

Table 1. $\log P$, Hydrolysis Half-Lives, and Antiviral Activity Data of Different *cyclo*Sal-d4TMP Triesters **15**

Comp.	Subst. X	$\log P$ value ^a	Hydrolysis ($t_{1/2}$) in aqueous buffers at 37 °C			Hydrolysis ($t_{1/2}$) in CM ^f at 37 °C		Antiviral activity EC ₅₀ (μM) ^h			Cytotoxicity CC ₅₀ (μM) ⁱ
			pH 6.9 ^b [h] ^e	pH 7.3 ^c [h] ^e	pH 8.9 ^d [h] ^e	without FCS ^g [h] ^e	with FCS ^g [h] ^e	CEM/O HIV-1	CEM/O HIV-2	CEM/TK ⁻ HIV-2	
15a	5-NO ₂	0.17	4.1	0.15	0.06	n.d.	0.12	0.29	0.40	40.0	75
15b	5-Cl	0.88	6.4	0.7	0.3	2.5	1.0	0.42	1.40	2.67	49
15c	5-H	0.28	24.5	4.5	1.1	7.2	3.9	0.28	0.10	0.50	47
15d	5-OMe	0.36	28.3	7.2	1.07	7.2	4.3	0.18	0.70	0.80	39
15e	5-Me	0.73	28.3	8.0	1.3	8.9	8.4	0.18	0.34	0.18	38
15f	5-sBu	1.94	n.d.	14	n.d.	n.d.	n.d.	0.19	0.85	1.90	47
15g	5- <i>t</i> Bu	1.70	n.d.	16	n.d.	n.d.	n.d.	0.14	0.90	1.5	34
15h	3.5-Me	1.18	98.2	32	3.4	17.5	10.8	0.09	0.17	0.08	21
15i	3-OMe	0.14	9.5	1.4	0.4	3.2	2.0	0.17	0.42	0.17	35
15j	3-Me	0.70	68.5	24	1.5	16.0	9.1	0.057	0.07	0.048	26
15k	3- <i>i</i> Pr	1.60	n.d.	28	n.d.	n.d.	n.d.	0.047	0.08	0.065	18
15l	3-sBu	1.83	n.d.	36	n.d.	n.d.	n.d.	0.08	0.12	0.09	18
15m	3- <i>t</i> Bu	1.75	n.d.	96	n.d.	n.d.	n.d.	0.18	0.15	0.18	49
d4T 1	–	–0.82	n.a.	n.a.	n.a.	n.a.	n.a.	0.25	0.15	50	49

^a $\log P$: partition coefficient;^bpH 6.9: 30 mM TRIS buffer;^cpH 7.3: 30 mM sodium phosphate buffer;^dpH 8.9: 30 mM sodium borate buffer;^eHalf-lives are given in hours;^fCM: RPMI-1640 culture medium, 37 °C;^gFCS: 10% heat-inactivated fetal calf serum;^h50% effective concentration;ⁱ50% cytotoxic concentration;

n.d.: not determined; n.a.: not available.

to design a delivery mechanism that is independent to enzymatic activation. However, the product distribution was slightly different as compared to the chemical hydrolysis studies. In addition to the nucleotide d4TMP, also d4T was observed, which is due to the enzymatic dephosphorylation of d4TMP by cell extract phosphatase activity. Further studies in human serum (10% serum in phosphate buffer) showed no difference in stability as compared to the buffer hydrolysis studies.⁴⁹ Hence, no enzymatic contribution could be detected. The donor-substituted *cycloSal*-d4TMPs **15d–h, j–m**, which exhibited half-lives of 7–99 h, should be interesting candidates for biological evaluation. Consequently, by playing around with the electronic properties of the aryl substituents we were able to adjust a certain hydrolytic stability (Table 1). As the NMR-experiment, all data that we found in the hydrolysis studies are in perfect agreement with the designed degradation pathway according to the tandem-reaction mechanism of the *cycloSal*-phosphate triesters.

It was interesting to have a closer look on the second step of the degradation pathway: the cleavage of the intermediate benzyl phosphate diester **16**. Therefore, an experiment in ¹⁸O-labeled water was carried out.³⁵ The first step had to be a nucleophilic attack at the phosphorus atom. This led to a benzyl phosphate diester that bears one ¹⁸O-label at the phosphorus center. The next step leading to the nucleotide d4TMP and the salicylalcohol **18** may proceed by three different mechanisms:

- (i) through a second nucleophilic attack at the P-atom (which is unlikely);
- (ii) *via* a spontaneous C–O-bond cleavage in the benzyl ester linkage (and formation of a benzyl cation that is then quenched by water) and d4TMP or finally,
- (iii) *via* a nucleophilic attack at the benzyl-carbon atom under displacement of d4TMP (that leads directly to the salicylalcohol) (Figure 10).

The outcome of this experiment with respect to the ¹⁸O-label would be the following: the first possibility (i) would lead to *two* ¹⁸O-labels within d4TMP and no label in the masking group; the second and the third scenario would lead to *one* ¹⁸O-label in both d4TMP and the salicylalcohol **18**. This experiment cannot distinguish between possibility (ii) and (iii). The hydrolysis mixture was then subjected to electrospray-mass spectrometry (ESI-MS). From the spectra it became apparent that mass peaks with M + 2 for d4TMP and M + 2 for the salicylalcohol

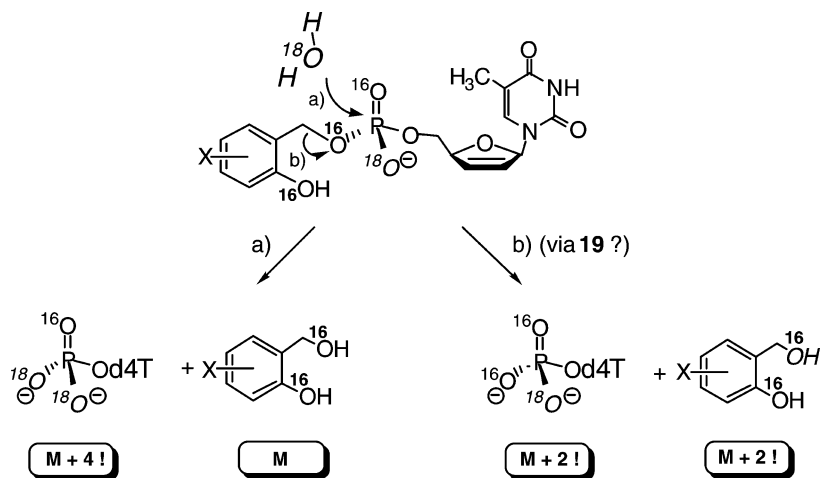


Figure 10. Mass spectrometric analysis of the hydrolysis mixture from ^{18}O -labeled water.

18 were observed. This analytical observation clearly excludes the first possibility (i) because in this case the d4TMP peak should have a mass of $M + 4$ (Figure 10).

So, no nucleophilic attack at the P-atom takes place and—as a consequence—the pseudorotation phenomenon¹⁷ could definitely be excluded. This is also the reason why we never observed the coformation of the parent nucleoside d4T in the chemical hydrolysis samples. At the same time, this experiment proves that the masking group is intrinsically activated due to the first hydrolysis step because of the “Umpolung” of the substituent. Our newest interpretation for the very rapidly progressing second degradation step includes an intramolecular proton-transfer from the phenol oxygen to the benzyl ester oxygen in **16** with the result of a high increase in leaving group tendency of the phosphate ester d4TMP (Figure 11).

This would explain why “normal” donor substituted benzyl phosphate triesters as **9** cleave only one benzyl group to yield the phosphate diester **11** and did not further hydrolyze because the phosphate group is negatively charged and therefore it is a bad leaving group (Figure 6b).

Finally, the cycloSal-d4TMP triesters **15** as well as triesters bearing other nucleoside analogues showed a marked increase in lipophilicity (experimentally determined log P value in 1-octanol/water,⁵⁰ Table 1) with respect to d4T (and in general with respect to the parent nucleo-

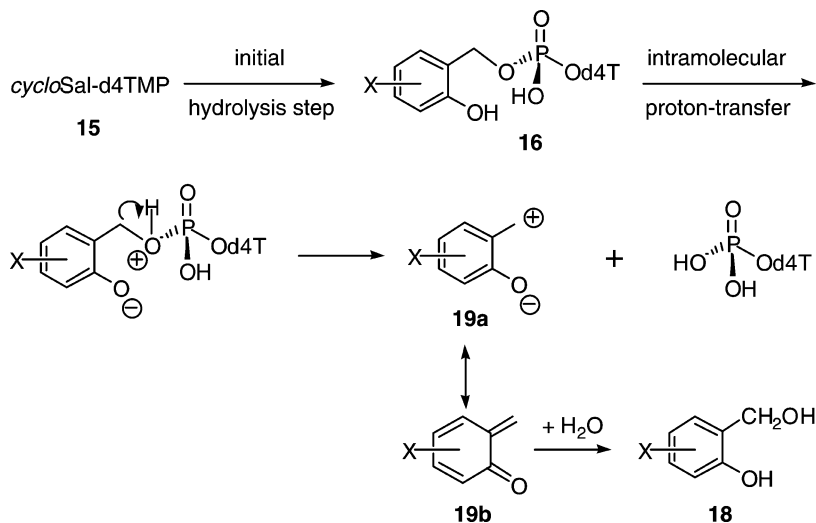


Figure 11. Intramolecular leaving group activation by proton-transfer.

sides). This may point to a higher potential of passive diffusion through the cellular membranes and through the blood–brain barrier.^{50a,51} Despite of the increased lipophilicity, it should be emphasized that *cycloSal* triesters are still reasonably water-soluble.

V. PROOF-OF-PRINCIPLE FROM ANTIVIRAL ASSAYS

The effectiveness of the *cycloSal*-nucleotides was demonstrated in *in vitro* antiviral assays.³⁵ For *cycloSal*-d4TMPs **15**, a correlation of the structure and the biological activity as in the hydrolysis studies was found: the stronger the electron-donating activity of the substituent, the better the antiviral activity against HIV-1 and HIV-2 in CEM cells. The 3- (**15j–m**), 5-alkyl- (**15e–g**) as well as the 3,5-dimethyl-*cycloSal*-d4TMP **15h** exhibited comparable or even higher antiviral potency (0.087 μ M) than d4T **1** (0.18 μ M, Table 1). Quite striking was the maintenance of the biological activity in thymidine kinase-deficient CEM/TK[−] cells for most of the compounds, particularly the 3-alkyl-substituted compounds (Table 1). Only the hydrolytically most labile 5-nitro-*cycloSal*-d4TMP (**15a**, $t_{1/2}$ 0.15 h) and to some extent the 5-chloro-derivative (**15b**, $t_{1/2}$ 0.7 h) showed a complete loss in antiviral activity. This points to a preferential extracellular hydrolysis to yield d4TMP which would then be hampered in its cellular uptake due to its

Table 2. Anti-HIV Activity Data of the *cyclo*Sal-d4TMP Triesters **15** in MT-4 Cells

Comp.	Subst. X	Config.	HPLC ^b	Antiviral activity EC ₅₀ (μM) ^a	
				MT-4 HIV-1	MT-4 HIV-2
15b	5-Cl	R _p /S _p	mixture	0.031	0.038
15e	5-Me	R _p /S _p	mixture	0.021	0.029
15h	3,5-Me	R _p	slow	0.025	0.026
15h	3,5-Me	S _p	fast	0.066	0.080
15j	3-Me	R _p /S _p	mixture	0.028	0.033
15j	3-Me	R _p	slow	0.012	0.011
d4T 1	—	—	—	0.018	0.022

^a 50% effective concentration;^b Eluting properties of the compound on reversed phase HPLC column.

high hydrophilicity. From the antiviral data and the hydrolysis half-lives it became apparent that we need certain stability, but, if that is reached, no further correlation is observed. This can be seen, e.g., from the comparison of the 3-methyl- (**15j**) and the 3-*t*-butyl-*cyclo*Sal-d4TMP (**15m**). Additionally, the high biological activity of the donor-substituted *cyclo*Sal-d4TMP derivatives was also observed in MT-4 and Molt4/C8 cells³⁵ (Table 2).

These results confirm the uptake of the compounds into the cells, the highly selective intracellular delivery of d4TMP, and the independence of the latter on the cellular thymidine kinase activation for the expression of the antiviral activity. The compounds thus achieve an efficient TK-bypass.

Interestingly, although we have determined considerable differences in the lipophilicity of the different *cyclo*Sal-d4TMPs, no correlation with the antiviral activity could be deduced. This can be seen in Table 1, where 3-methyl-*cyclo*Sal-d4TMP **15j** (log*P* 0.70) is compared with 3-*s*Bu-*cyclo*Sal-d4TMP **15l** (log*P* 1.83). Although these compounds showed a 13-fold difference in lipophilicity, the antiviral activity was very similar. However, the *in vitro* anti-HIV test gives only an indirect proof of the intracellular delivery of d4TMP. Therefore, we did further incubation experiments with wild-type CEM and CEM/TK[−] cells using the radiolabeled 3-methyl-*cyclo*Sal-d4TMP **15j** (tritium-label in the methyl group of thymine).^{52,53} As can be seen in Table 3, the amount of detected d4TMP in CEM/O cells was considerably higher as compared to the amount of d4TMP resulting from the metabolism of d4T in the same cell line. The same holds for the concentration of d4TTP in CEM/O cells that may explain in part the higher activity

Table 3. Cell Incubation Studies Using Radiolabeled 3-Methyl-*cyclo*Sal-d4TMP **18j** and AZTMP **29e** as well as their Parent Nucleosides

Compound	Incub. time	CEM/O cells pmol/10 ⁹ cells				Mutant CEM/TK ⁻ cells pmol/10 ⁹ cells			
		N + triester	NMP	NDP	NTP	N + triester	NMP	NDP	NTP
d4T 1 ^a	24	110	13	3.0	37	8.2	0.3	0.3	1.5
3Me- <i>cyclo</i> Sal-d4TMP ^b	24	85	93	19	296	55	32	14	236
R _p -3Me- <i>cyclo</i> Sal-d4TMP ^c	24	56	91	7.6	151	n.a.	n.a.	n.a.	n.a.
S _p -3Me- <i>cyclo</i> Sal-d4TMP ^c	24	45	16	1.8	36	n.a.	n.a.	n.a.	n.a.
R _p /S _p -mixture ^c	24	79	49	5.1	101	n.a.	n.a.	n.a.	n.a.
AZT 2 ^d	2	80	10990	313	816	b.d.	b.d.	b.d.	0.1
AZT 2 ^d	6	112	16370	412	1032	b.d.	1.0	b.d.	0.3
AZT 2 ^d	24	84	9473	427	843	b.d.	0.5	n.d.	1.1
R _p /S _p -3Me- <i>cyclo</i> Sal-AZTMP ^e	2	12	171	13	11	n.a.	6.7	2.2	4.0
R _p /S _p -3Me- <i>cyclo</i> Sal-AZTMP ^e	6	13	1215	57	146	n.a.	n.a.	n.a.	n.a.
R _p /S _p -3Me- <i>cyclo</i> Sal-AZTMP ^e	20	39	5475	236	425	n.a.	2.0	1.1	1.4
R _p /S _p -3Me- <i>cyclo</i> Sal-AZTMP ^e	44	n.a.	n.a.	n.a.	n.a.	n.a.	1.1	b.d.	0.3

^a0.1 μM d4T **1**;^b0.1 μM 3-Me-*cyclo*Sal-d4TMP **15j**;^c0.17 μM of each triester was used;^d0.09 μM AZT **2**;^e0.07 μM R_p/S_p-3-Me-*cyclo*Sal-AZTMP **29e**;

n.a.: not available; n.d.: not determined; b.d.: below detection limit.

of the *cycloSal*-d4TMP triesters in wild-type CEM cells as compared to d4T. More striking were again the studies in CEM/TK⁻ cells. While d4T was not metabolised to give the phosphorylated derivatives, the concentrations of d4TMP as well as d4TTP generated from the pronucleotide and forward phosphorylation were quite high. These studies perfectly explain the outcome of the results of the antiviral tests of the *cycloSal*-d4TMPs **15** and again prove the TK-bypass strategy.

As can be seen from the structure of the *cycloSal*-d4TMP triesters **15**, the phosphorus atom is a stereogenic center. Due to the fact that the chemical synthesis proceeds without any diastereoselectivity the resulting *cycloSal*-phosphate triesters were isolated as 1:1 diastereomeric mixtures. It cannot be excluded that these different diastereomers possess different properties with respect to their hydrolytic behavior as well as their antiviral activity. Therefore, the diastereomeric mixtures were separated by semipreparative HPLC. However, no crystallisation of the phosphate triesters was successful that would provide an excellent method to assign the stereochemistry at the phosphorus atom (X-ray analysis). Instead, we were successful to define the stereochemistry indirectly by correlation of the elution properties on a RP-18 silica gel column, ³¹P-NMR chemical shifts and, finally, antiviral activity. This correlation was possible because we prepared the two diastereomers of the corresponding *cycloSal*-(–)-menthylmonophosphates **28**.⁵⁴ They were separated and crystallized, and structure determination by X-ray analysis revealed the absolute configuration at the phosphorus atom (Figure 12). CD-spectroscopy showed then the expected two specific inverted CD bands due to the Cotton-effect at 224 nm (Figure 12). The correlation of the configuration and the sign of the Cotton-effect have been used for the configuration assignment of the P-atom in the separated *cycloSal*-d4TMP triesters **15**.⁵⁵ A negative Cotton-effect at 224 nm was caused by R_p-configuration, while a positive effect correlated with the S_p-configuration. With this information we correlated the ³¹P-NMR chemical shifts of the *cycloSal*-d4TMP triesters and these with the antiviral activity (Figure 13).³⁵

In chemical hydrolysis studies we observed a two-fold difference in the chemical stability of the diastereomers of 3-methyl-*cycloSal*-d4TMP **15j**.³⁵ This may be due to the different position of the substituents at the half-chair formed by the 4H-benzodioxaphosphorin-2-oxide part of the triesters. It has been shown previously that different position of the substituents (axial or equatorial) in a half-chair may influence the chemical hydrolysis properties of the phosphate ester bonds.³²

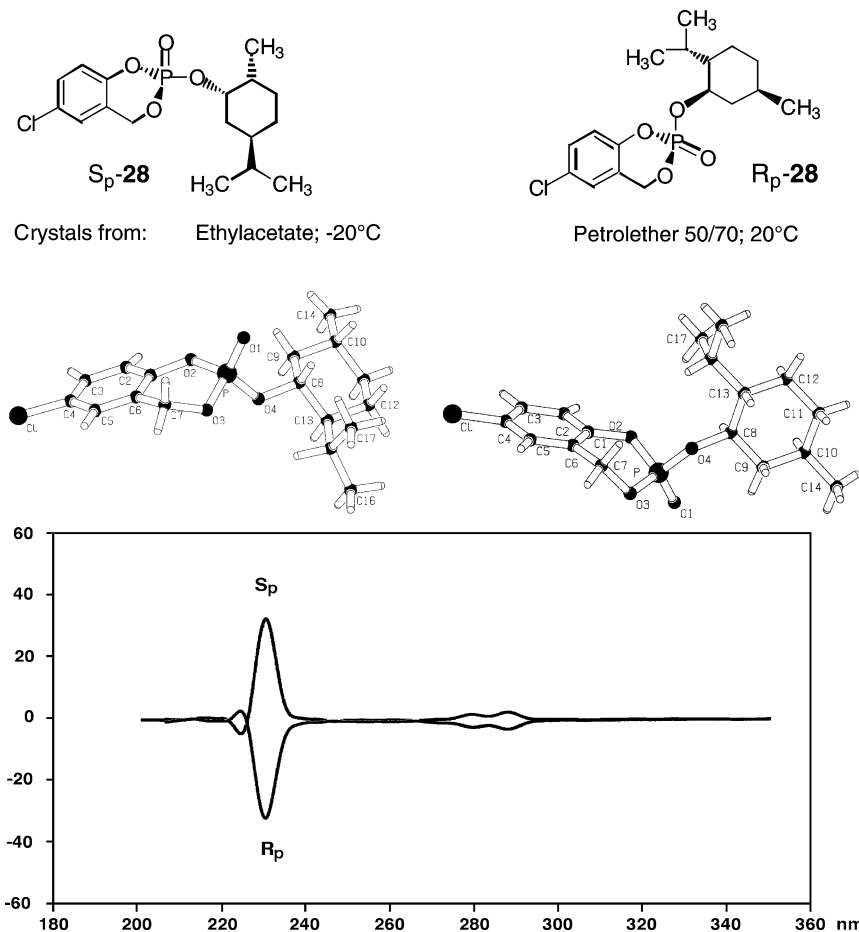


Figure 12. Formula, crystal structure, and CD-spectra of R_p- and S_p-5-chloro-cyclo-Sal-(-)-menthyl monoposphate **28**.

From the antiviral evaluation of the separated diastereomers it became apparent that both stereoisomers showed different antiviral activity. The R_p-configured stereoisomer ("slow"-eluting) was invariably more potent than the S_p-stereoisomer ("fast"-eluting). The difference in activity was about 5- to 10-fold either in TK-competent CEM/O cells as well as in mutant TK-deficient CEM cells (Table 4). Nevertheless, even the S_p-stereoisomers retained the antiviral activity in the TK-deficient cells. The same has been observed in MT-4 cells infected with either HIV-1 or HIV-2 (Table 2).³⁵

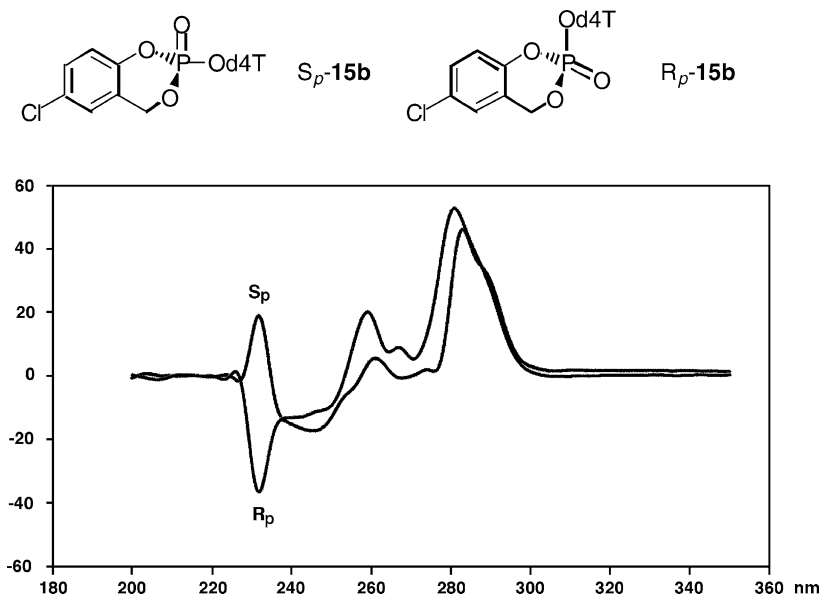


Figure 13. Formula and CD-spectra of *R_p*- and *S_p*-5-chloro-cycloSal-d4TMP.

As before, for both diastereomers of 3-methyl-cycloSal-d4TMP **R_p/S_p-15j** in their tritium labeled form we monitored their intracellular fate in CEM/O cells. In these studies it was clearly shown that the *R_p*-configured phosphate triesters generated about 10-fold higher d4TMP levels and (thus) also about five-fold higher d4TTP level as compared to the *S_p*-triesters (Table 3).⁵² This perfectly explains the observed difference in the (indirect) antiviral activity assay. If this difference in intracellular nucleotide pool is relying (only) on the difference in chemical hydrolysis stability remains to be evaluated.

Moreover, the cycloSal-d4TMP triesters demonstrated significant antiviral activity in AZT-resistant H9^rAZT²⁵⁰ cells, which proves that the compounds seem to be able to overcome cellular resistance to a certain drug. The resistant cell line was generated by continuous cultivation of H9 cells in the presence of increasing AZT concentrations. The developed AZT resistance was associated with a five-fold lower expression of the TK gene in comparison to the parental H9 cells.^{10a} Consequently, AZT showed an EC₅₀ of > 100 μM in this cell subline, while the EC₅₀ in parental cells was found to be 0.04 μM. In addition, also d4T lost most of its activity in the H9^rAZT²⁵⁰ cells as compared to the parental H9 cells (EC₅₀: 26 and 0.9 μM, respectively). CycloSal-

Table 4. Stereochemical Dependence of Antiviral Data of the *cyclo*Sal-d4TMP Triesters **15**

Comp.	Subst. X	Mobility in RP-HPLC ^a	³¹ P-NMR (ppm) ^b	Antiviral activity EC ₅₀ (μM) ^c			Cytotoxicity CC ₅₀ (μM) ^d	SI value ^e
				CEM/O HIV-1	CEM/O HIV-2	CEM/TK ⁻ HIV-2		
R _p - 15e	3- <i>t</i> Bu	fast	−8.62	0.13	0.44	0.19	25	190
S _p - 15e	3- <i>t</i> Bu	slow	−8.43	0.6	3.0	5.0	79	16
R _p - 15g	5- <i>t</i> Bu	fast	−7.85	0.09	0.8	0.9	27	30
S _p - 15g	5- <i>t</i> Bu	slow	−7.83	0.21	0.65	7.5	33	5
R _p - 15h	3,5-Me	slow	−7.32	0.093	0.17	0.08	18	218
S _p - 15h	3,5-Me	fast	−7.63	0.50	0.80	0.38	22	57
R _p - 15i	3-OMe	slow	−7.82	0.14	0.17	0.12	38	320
S _p - 15i	3-OMe	fast	−7.81	0.47	1.03	9.67	84	9
R _p - 15j	3-Me	slow	−7.49	0.08	0.067	0.063	11	190
S _p - 15j	3-Me	fast	−7.69	0.42	1.1	0.70	76	108
d4T 1	—	—	—	0.18	0.26	15.0	56	4

^a Eluting properties of the compound on reversed-phase HPLC column;^b ³¹P-NMR chemical shift in DMSO-d₆;^c 50% effective concentration;^d 50% cytotoxic concentration;^e Selectivity index: ratio 50% cytotoxic concentration/50% effective concentration.

d4TMP **15c** proved to be equipotent in parental and in H9^rAZT²⁵⁰ cells (EC₅₀: 0.3 and 0.5 μ M, respectively).⁵⁶ However, cytotoxicity of d4T was comparable in AZT-resistant and in parental cells. These results indicate that HIV-1-infected cells may develop antiviral resistance towards an anti-HIV drug without a concomitant decrease in the cytotoxicity of the drug.

Finally, it should be mentioned that all modifications of the *cycloSal*-structure except for the substituents in the aromatic ring led to a markedly loss in antiviral activity. In this context we prepared phosphoramidate derivatives in which the phenol oxygen was replaced by an amino or an N-methylamino fragment (*cycloAmb*-d4T phosphoramidates).⁵⁷ As compared to the original *cycloSal*-d4TMP triesters, the *cycloAmb*-phosphoramidates showed a considerable increase in hydrolytic stability that was associated with a complete loss in antiviral activity. Modification of the benzyl position, introducing alkyl groups (methyl, butyl, and functionalized alkyl groups), led, surprisingly, to a complete inversion of the bond cleavage selectivity for the former two alkyl groups.⁵⁸ Now the benzyl ester bond, instead of the desired phenol ester bond, was selectively broken. Consequently, the modified phenyl phosphate diester **17** was formed as the main product. As expected, no further degradation to yield d4TMP was observed. The picture was much more complicated for the ester-functionalized *cycloSal*-d4TMP which showed a complex reaction profile leading to the modified phenyl phosphate diester **17** but also to the modified benzyl phosphate diester **16**. The latter compound was further hydrolyzed so as to give d4TMP. The ratio of the concurrent reactions could be influenced by the aryl ring substituent. Electron-withdrawing substituents favor the formation of the benzyl phosphate diester, while electron-donating groups push the reaction in direction of the “false” phenyl phosphate diester. However, high amounts of d4TMP could be obtained to the expense of hydrolytic stability. Consequently, also these derivatives virtually lost the antiviral activity as compared to the prototype *cycloSal*-d4TMP triesters. Nevertheless, some of the compounds still showed promising antiviral activity in the TK-deficient CEM cells that was clearly superior to that of d4T.⁵⁸

VI. THE FAILURE OF THE *cycloSAL*-AZTMP TRIESTERS

In addition to the dideoxynucleoside analogue d4T **1**, the *cycloSal* approach has also been applied to AZT **2**.^{59,60} The preparation of these

Table 5. Anti-HIV-Data of the *cyclo*Sal-AZTMP Triesters **29** in CEM Cells

Comp.	Subst. X	log <i>P</i> value ^a	Antiviral activity EC ₅₀ (μM) ^b			Cytotoxicity CC ₅₀ (μM) ^c	SI value ^d
			CEM/O HIV-1	CEM/O HIV-2	CEM/TK ⁻ HIV-2		
29a	5-NO ₂	0.81	0.008	0.02	> 100	79	9800
29b	5-H	1.12	0.004	0.005	> 20	68	17 000
29c	5-Me	1.49	0.005	0.006	21	79	15 800
29d	3,5-Me	1.81	0.007	0.017	7	40	5700
29e	3-Me	1.43	0.006	0.013	15	40	6600
AZT 2	–	0.037	0.006	0.005	> 100	> 100	> 16 600

^a log *P*: partition coefficient in 1-octanol/water;
^b 50% effective concentration;
^c 50% cytotoxic concentration;
^d Selectivity index: ratio 50% cytotoxic concentration/50% effective concentration.

compounds (**29**) was done by the chlorophosphite method. From chemical hydrolysis studies we knew that these triesters behave as the corresponding *cyclo*Sal-d4TMP compounds: they release selectively AZTMP with half-lives that were slightly higher as compared to the d4TMP pronucleotides. The antiviral testing of these compounds showed a pronounced activity in the wild-type CEM cells against both HIV-1 and HIV-2. However, the *cyclo*Sal-AZTMP derivatives **29** lost almost their entire antiviral activity in mutant thymidine kinase-deficient CEM/TK⁻ cells. Nevertheless, a few of the compounds were still quite more active than the parent nucleoside AZT **2** (Table 5).⁶⁰

Conceptionally this was a surprising result because this mutant cell line is unable to phosphorylate AZT into its monophosphate and therefore we expected again the retention of antiviral activity. However, it is known that the metabolic bottleneck in the activation of AZT into its triphosphate is the conversion of AZTMP into AZTDP.^{3a,5,8a,61} Consequently, it seems that there is a special metabolic limitation that is acting against the retention of activity in the TK⁻ cells. To investigate the reasons for the failure of the *cyclo*Sal-AZTMP derivatives **29**, again a study using radiolabeled 3-methyl-*cyclo*Sal-AZTMP R_p/S_p-**29e** was conducted.⁶² From studies in CEM/O cells and CEM/TK⁻ cells it became apparent that the amount of AZTMP found in the wild-type CEM cells incubated with the *cyclo*Sal-AZTMP triester was considerably lower as compared to the AZTMP concentration found in the same cell line incubated with AZT itself (Table 3). Only after 48 h of incubation the AZTMP levels formed from the triester reached the same level

as those formed from the nucleoside AZT. However, in CEM/TK⁻ cells AZT formed extremely low levels of AZTMP (slightly over detection limit), while the levels formed by hydrolysis of the *cyclo*Sal-AZTMP triester were considerably higher. This at least explains again why the compounds kept some antiviral activity in contrast to AZT. Nevertheless, the comparison of the AZTTP levels formed from the triester in CEM cells and in mutant CEM/TK⁻ cells showed the expected difference: there were 100-fold decreased AZTTP levels after 6 h of incubation in the mutant cell line (Table 3). This explains the 100-fold decrease in antiviral activity in the mutant cell line as compared to "normal" CEM cells.

Our interpretation of the failure of the AZTMP triesters **29** is the following: due to the fact that the bottleneck in the activation of AZT is the conversion of AZTMP into AZTDP an increase of the AZTMP level by release from a pronucleotide would act contraproductive for the forward phosphorylation. Assuming an efficient enzymatic dephosphorylation of AZTMP into AZT, the intracellular delivery of AZTMP cannot improve the activity. AZT formed in wild-type cells by this catabolic reaction is readily re-phosphorylated to AZTMP by the cellular thymidine kinase (TK) and, so, one would not observe a severe effect on the antiviral activity (as we indeed found). In contrast, in mutant CEM/TK-deficient cell this process would have severe consequences: if the released AZTMP from the pronucleotide is readily dephosphorylated, the intracellular pool of AZTMP is dramatically reduced because no thymidine kinase phosphorylation restores the AZTMP pool. This dephosphorylation/rephosphorylation metabolism only plays a significant role in the case of AZT because of the limited forward phosphorylation of AZTMP in TK⁻ cells (Figure 14). An identical process does not account in the case of d4T because the bottleneck within the metabolism is the formation of d4TMP by the cellular TK and the d4TMP formed from the pronucleotide is readily forward phosphorylated into d4TDP (Figure 15).

An enzyme that may play a role in the dephosphorylation of AZTMP is 3',5'-(deoxy)nucleotidase. From kinetic studies using this enzyme it was shown that the relative efficiency for the dephosphorylation of dTMP, d4TMP, and AZTMP was 1, 0.08, and 2, respectively. This shows that AZTMP is even a better substrate for this enzyme than dTMP, while d4TMP is about 20-fold less efficiently converted into d4T than AZTMP and 10-fold less so than dTMP. This study may point to an important factor when pronucleotides are used in order to bypass metabolic limitations: the selective release of the nucleotide may not be sufficient to overcome a limitation if unknown catabolic processes are

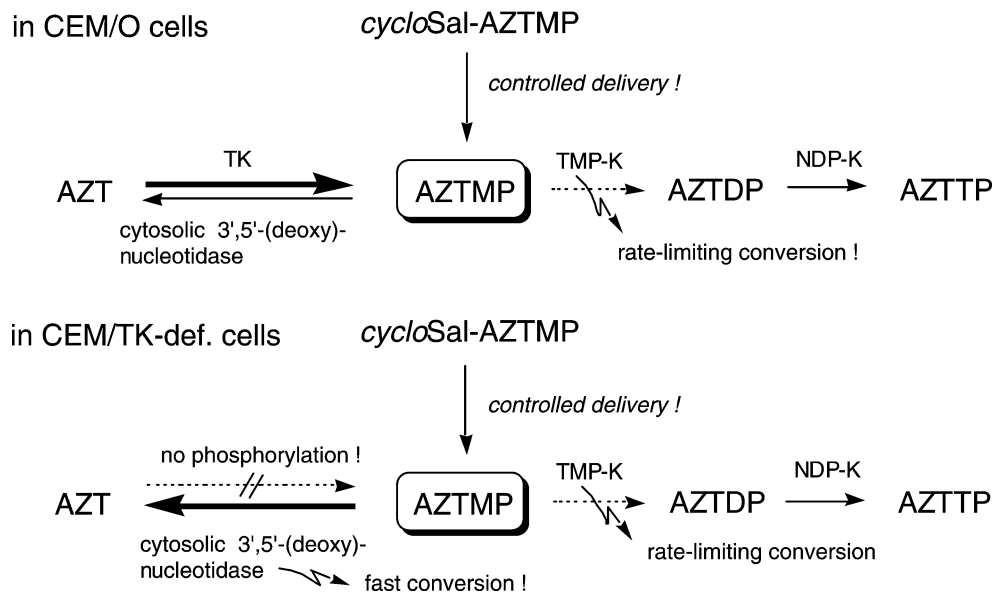


Figure 14. Comparative metabolism of *cycloSal-AZTMP* **29** in CEM/O and CEM/TM⁻ cells.

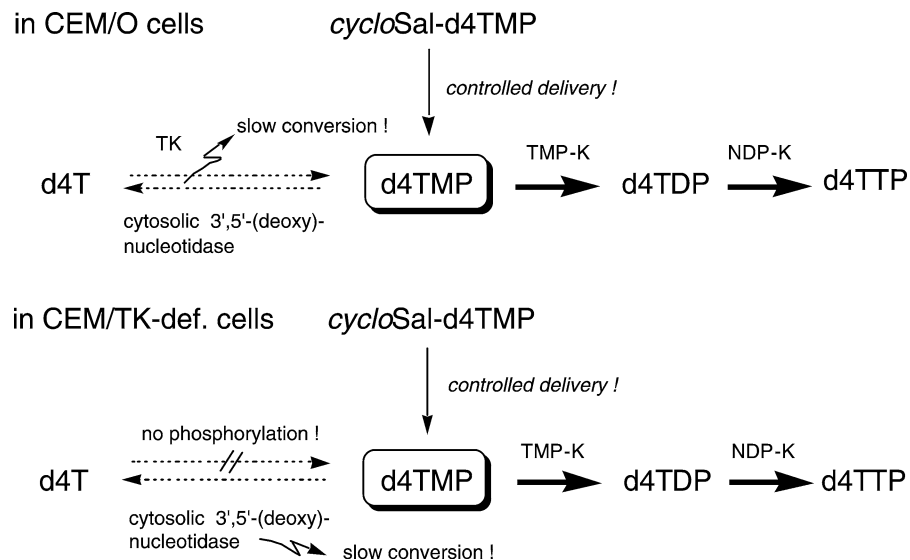


Figure 15. Comparative metabolism of *cycloSal*-d4TMP **15** in CEM/O and CEM/TM⁻ cells.

taking place in parallel. We are now investigating the importance of the mentioned enzyme in dephosphorylation reactions in more detail.

Finally, it should be mentioned that all other pronucleotides of AZTMP reported by McGuigan et al.,⁶³ Imbach et al.,⁶⁴ Wagner et al.,⁶⁵ and Pompon et al.⁶⁶ also lost most of their activity in the thymidine kinase-deficient cell line. This clearly points to an intrinsic problem during AZT metabolism and not to a general problem of the *cycloSal*-pronucleotide concept because the mentioned delivery concepts are entirely different in this case.

VII. *cycloSAL*-PRONUCLEOTIDES OF 5-FLUORO-2'-DEOXYURIDINE (FDU)

In antitumor chemotherapy normally only the heterocycle 5-fluorouracil (5-FU) is used. However, the mechanism of action of this compound includes the intracellular formation of 5-fluoro-2'-deoxyuridine monophosphate (FdUMP).⁶⁷ FdUMP needs not to be further phosphorylated because it acts as such as an inhibitor of thymidylate synthase that is responsible for the methylation of dUMP into dTMP. It appeared reasonable to apply the *cycloSal*-concept (compounds **30** starting from FdU **3**) to the direct intracellular delivery of FdUMP.⁶⁸

The chemical synthesis was first carried out using the chlorophosphate method.^{68b} The regioselectivity can be controlled only moderately: we obtained a 2:1 selectivity for the 5'-O-phosphorylated product **30** and the 3', 5'-O-bisphosphorylated side product. However, using the phosphorus(V)-route (Figure 8) the regioselectivity increases to 5:1 in favor of the 5'-O-phosphorylated compound **30**.^{68a} Interestingly, only a very minor amount of the also possible 3'-O-phosphorylated *cycloSal*-FdUMP triester could be isolated. This led to the conclusion that the first 5'-O-phosphitylation resulted in an intramolecular activation of the 3'-hydroxyl group with a second reaction at the C3'-oxygen atom. Additionally, we tried to block the 3'-hydroxyl group by levulinylation (Lev-group). However, although the synthesis of the corresponding *cycloSal*-triesters proceeded in excellent yields, we were unable to cleave the Lev-group by hydrazine hydrate treatment. Only degradation of the heterocyclic ring was observed. It was interesting to note that the *cycloSal*-FdUMP triesters **30** were easily separable by semipreparative HPLC to yield both diastereomers. This allowed correlation studies concerning stereochemistry, ³¹P-NMR chemical shift and elution properties on HPLC.

The separated diastereomers of the *cyclo*Sal-FdUMP phosphate triesters **30** were evaluated for their biological activity in the following cell lines: L1210/O, L1210/TK⁻, FM3A/O, FM3A/TK⁻, Molt4/C8, CEM/O, and CEM/TK⁻ (Table 6).^{68a} Surprisingly, in FM3A/O cells the triesters showed lower antitumor activity (0.057 μ M) than the parent nucleoside 5-FdU (0.009 μ M). Moreover, as for FdU itself, we observed a loss in antitumor activity in L1210/TK⁻ and FM3A/TK⁻ cells (IC₅₀ 2.5–5 μ M). However, in wild-type L1210 (0.004 μ M), Molt4/C8 (16 μ M) and in CEM cells (0.03 μ M), the biological activity of both the triesters **30** and the parent nucleoside analogue **3** proved to be equipotent. A considerable loss (1000- to 200-fold, respectively) in activity was observed for compounds **30** in L1210/TK⁻ and in CEM/TK⁻ cells as compared to the wild-type cell line (Table 6). Additionally, the antithymidylate synthase activity was determined in a specific inhibition assay.⁶⁹ The *cyclo*Sal-FdUMP triesters **30** proved to be 10-fold less efficient than FdU, and this was independent of the use of L1210/O cells or mutant L1210/TK⁻ cells (data not shown).

It should be mentioned that in the CEM/O assay 5-FU showed an IC₅₀ of 0.7 μ M, which means that 5-FU was 23-fold less active than the *cyclo*Sal-compounds **30**.

Nevertheless, taking all together, from these data it is obvious that no efficient nucleotide delivery took place. However, having the results of the corresponding AZTMP compounds in mind, we cannot exclude that again a competing dephosphorylation takes place resulting in a fast intracellular clearance of FdUMP released from the pronucleotide. For this reason we will study if FdUMP is a good substrate for 3',5'-(deoxy)nucleotidase. Furthermore, another possible limitation may be the cellular uptake of the triesters. Evidence for this assumption may be taken from the following experiment: the aforementioned thymidylate synthase assay has been repeated in permeabilised L1210/O cells.⁶⁹ As test compounds FdU **3**, FdUMP, and 5-Cl-*cyclo*Sal-FdUMP **30a** were used. The IC₅₀ values were 2.50, 0.15, and 0.30 μ M, respectively. Here, the triester shows clearly a comparable biological activity as FdUMP and a higher potency as compared to FdU **3**. This experiment proves that FdUMP and not FdU **3** is released from the pronucleotide, and indirectly points to possible problems during uptake in non-permeabilised cells. A further interpretation should be discussed briefly. In contrast to the anti-HIV nucleoside analogues used so far, FdU possesses a hydroxyl group at the 3'-position and therefore an internal nucleophile. This hydroxyl group may attack instead of the external water nucleophile the phosphorus atom which would still lead to the cleavage of the *cyclo*Sal mask but under formation of 3',5'-cyclic

Table 6. Antitumor Activity of the *cyclo*Sal-FdUMP Triesters **30** in Different Cell Lines

Comp.	Subst. X	Antiviral activity IC ₅₀ (μM) ^a						
		L1210/0 ^b	L1210/TK ^{-b}	FM3A/0 ^b	FM3A/TK ^{-b}	Molt4/C8 ^c	CEM/0 ^c	CEM/TK ^{-c}
30a	5-Cl	0.0041	4.3	0.077	3.00	20.2	0.054	10.9
30b	H	0.0057	4.2	0.012	3.07	20.3	0.070	≥ 5.7
30c	5-OMe	0.0044	4.2	0.073	3.71	16.6	0.065	≥ 5.1
30d	5-Me	0.067	4.9	0.13	3.96	19.2	0.049	11.4
30e	3,5-DiMe	0.0081	3.8	0.18	4.63	17.7	0.072	11.5
30f	3-OMe	0.0044	3.8	0.057	2.65	24.4	0.071	12.2
30g	3-Me	0.0077	4.9	0.12	3.25	20.9	0.029	4.85
FU	–	–	–	–	–	–	0.7	–
FdU 3	–	0.0034	3.0	0.0096	1.98	15.5	0.058	4.22

^a 50% Inhibitory effect;^b Murine leukemia cells;^c Human T-lymphocyte cells.

FdUMP (cFdUMP) (see analogous reaction in Figures 23 and 25). This cyclic diester can not act as an inhibitor of thymidylate synthase. However, all data obtained from chemical hydrolysis studies exclude the formation of cFdUMP (co-elution on RP-HPLC with an independently synthesized authentic sample of 3',5'-cyclic FdUMP).⁵⁵ Moreover, the results using the permeabilized cells would contradict this interpretation.

It is interesting to note that other pronucleotides of FdUMP reported by McGuigan,⁷⁰ Imbach et al.,⁷¹ Farquhar et al.,⁷² Wagner et al.,⁷³ and Borch et al.⁷⁴ also lost most of their activity in the wild-type cells as well as the mutant cell lines. Again, this clearly points to an intrinsic problem during FdU metabolism.

VIII. ADA-BYPASS WITH *cyclo*SAL-ADENOSINE MONOPHOSPHATE DERIVATIVES

The *cyclo*Sal concept has also been successfully applied to different adenosine monophosphate derivatives: ddA **6**,^{75,76} the unsaturated 2',3'-dideoxy-2',3'-didehydroadenosine (d4A, **31**)⁷⁶ as well as 2',3'-dideoxy-2'-fluoro-*ara*-adenosine (F- β -ddA, **32**)⁷⁷ and 2',3'-dideoxy-2'-fluoro-*ribo*-adenosine (F- α -ddA, **33**).⁷⁷ Although the general structure and the delivery mechanism of the corresponding nucleotides is identical, the biological task of the compounds is different: while the d4T and AZT derivatives were designed to cause the TK-bypass, the mentioned adenosine analogues were designed for ADA-bypass. The metabolic fate of ddA has been studied in detail (Figure 16). DdA—and most probably the two fluorinated adenosine analogues mentioned above follow the same metabolic pathway—they are only poorly converted directly to the monophosphate by adenosine kinase or deoxycytidine kinase.⁷⁸ Indeed, starting from ddA **6**, only very low intracellular levels of ddATP are detected. This metabolite shows a similar inhibitory effect against HIV-RT ($K_i = 0.22 \mu\text{M}$) to that of AZTTP ($K_i = 0.1 \mu\text{M}$).^{6b} Furthermore, in marked contrast with the pyrimidine 2',3'-dideoxynucleosides (i.e., d4T, AZT), ddA **6** is extensively catabolized.⁷⁸ The main catabolic pathway is deamination into ddI **7** by the ubiquitous cellular enzyme adenosine deaminase (ADA).⁷⁹ The inosine derivative **7** is either inactivated by cleavage to hypoxanthine (Hx) by purine nucleoside phosphorylase (PNP), or further phosphorylated to the monophosphate ddIMP by 5'-nucleotidase,⁸⁰ a cellular enzyme whose activity remains nearly constant during all phases of the cell cycle.⁸¹

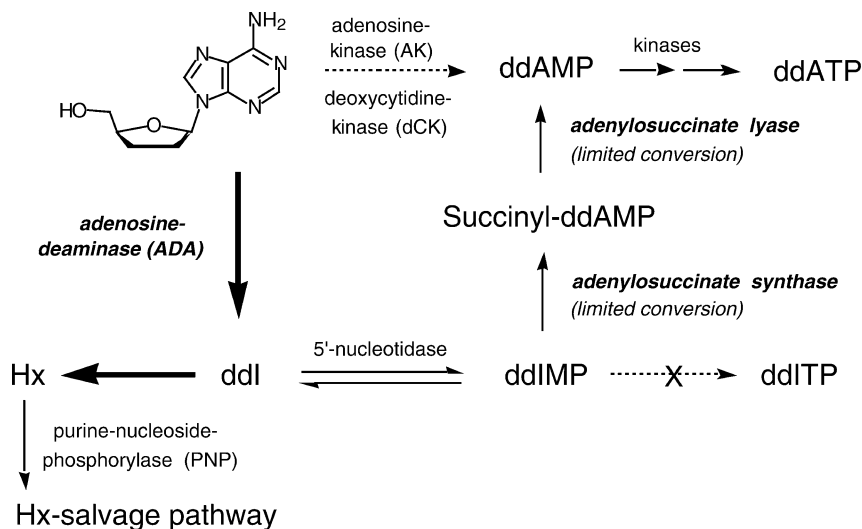


Figure 16. Metabolic fate of 2',3'-dideoxyadenosine (ddA).

No further phosphorylation of ddIMP to the corresponding triphosphate has been observed. Therefore, ddIMP enters the adenosine anabolic pathway through the action of adenylosuccinate synthetase and adenylosuccinate lyase to give 2',3'-dideoxyadenosine monophosphate (ddAMP).⁷⁹ However, the enzymatic conversion of ddIMP to ddAMP is not very efficient and both intervening enzymes become rate-limiting steps. Further phosphorylation by cellular kinases converts ddAMP to the triphosphate level (Figure 16).⁷⁸

Consequently, the intracellular delivery of ddAMP would bypass of at least four enzymatic reaction steps initiated by the ADA deamination.

The chemical synthesis of both the 3-methyl-*cyclo*Sal-ddAMP **34** and the 3-methyl-*cyclo*Sal-d4AMP triesters **35** were carried out using again our direct phosphitylation reaction using the chlorophosphites.⁷⁶ As expected, in all reactions we also obtained the N⁶-phosphorylated product (ca. 10% yield) besides the 5'-O-phosphorylated main product (50–60% yield). Due to the low solubility of the ddA **6** and d4A **31** in acetonitrile, a mixture of DMF/THF was used instead for the phosphitylation/oxidation reaction and the reaction temperature was lowered to –50 °C from –10 °C in order to increase the 5'-O- versus N⁶-regioselectivity.⁸² The use of protecting group chemistry for the exocyclic amino group of the adenine heterocycle was quite complicated

due to the highly acid lability of ddA and d4A and the base lability of the *cycloSal* mask at the glycon ring.

Again, chemical hydrolysis studies showed the selective delivery of ddAMP and d4AMP from the 3-methyl-*cycloSal*-triesters **34** and **35**, respectively.^{75,76} However, the half-lives proved to be significantly higher as compared to that of the corresponding *cycloSal*-d4TMP triesters.³⁵ As noted for the *cycloSal*-d4TMP triesters, the adenosine *cycloSal* triesters showed significantly increased log*P* values as compared to the parent nucleosides.⁸³ An important aspect on the clinical use of ddA and d4A is their dramatic acid sensitivity to acid catalyzed cleavage of the glycosidic bond.⁸⁴ Therefore, we studied the lability of the 3-methyl-*cycloSal*-modified ddAMP (**34**) or d4AMP triesters **35** at pH 3.0 and pH 1.0. To our surprise, upon introduction of the *cycloSal* masking group the stability increased 10-fold as compared to the free nucleoside analogue for some unknown reasons.

Next, the sensitivity of the 3-methyl-*cycloSal*-ddAMP and 3-methyl-*cycloSal*-d4AMP triesters **34**, **35** to deamination by ADA or adenosine monophosphate deaminase (AMPDA) was investigated. Incubation of the triesters with both enzymes demonstrated, as expected, a complete stability of the adenine heterocycle for 12 h (!), while the nucleosides were deaminated efficiently.⁸⁵ This observation is quite important for the interpretation of the biological data.

3-Methyl-*cycloSal*-ddAMP **34** exhibited high antiviral activity in CEM/0 cells (0.025 μM ; up to a 100-fold increase as compared to ddA **6** (4.3 μM , (Table 7)).^{75,76} In addition to the high activity, a 15-fold increase in the selectivity index (SI) was found. Moreover, the corresponding 3-methyl-*cycloSal*-d4AMP **35** proved to be 600-fold (0.05 μM) more potent as compared to d4A **31** (30 μM). The SI increased from 3 for the parent nucleoside d4A to up to 1300 for 3-methyl-*cycloSal*-d4AMP **35** (Table 7).⁷⁶ Again, these results point to an efficient delivery of the nucleotide ddAMP as well as of d4AMP. Due to the fact that the triesters were completely resistant to deamination, the biological activity is not caused by the direct delivery of ddIMP or d4IMP.

For comparison the corresponding ddIMP and d4IMP triesters **37** and **38**, respectively, were also prepared and tested.⁷⁶ It was interesting that 3-methyl-*cycloSal*-ddIMP **37** showed only an EC₅₀ value of 1.1 μM as compared to 0.025 μM for 3-methyl-*cycloSal*-ddAMP **34** and 4.3 μM for ddA **6** (Table 7). This verifies that the rate-limiting conversion from ddA to ddAMP is the enzymatic re-amination of ddIMP into ddAMP. 3-Methyl-*cycloSal*-d4IMP **38** showed an EC₅₀ value of 5.3 μM , while 3-methyl-*cycloSal*-d4AMP **35** proved 100-fold more active (0.05 μM) and d4I **36** was active at 4.3 μM . Again, enzymatic re-

Table 7. Antiviral Evaluation of 3-Methyl-*cyclo*Sal-Adenosine/Hypoxanthine Derivatives as Compared to their Parent Nucleosides

Comp.	Subst. X	Antiviral activity			Cytotoxicity		SI ^d
		EC ₅₀ (μM) ^a					
		CEM HIV-1	CEM HIV-2	C3H/3T3 MSV	CEM CC ₅₀ (μM) ^b	C3H/3T3 MCC (μM) ^c	
34	3-Me	0.047	0.03	1.18	28	> 50	585
ddA 6	–	4.3	4.5	40	> 250	> 50	> 57
36	3-Me	1.1	0.7	> 50	58	> 50	53
ddI 7	–	3.5	4.0	> 50	156	> 50	44
35	3-Me	0.065	0.19	1.24	49	> 50	746
d4A 31	–	30	50	35	96	> 50	3
37	3-Me	5.3	5.0	> 50	94	> 50	18
d4I 36	–	4.3	> 50	> 50	87	> 50	20
39	3-Me	3.67	3.3	n.d.	146	n.d.	44
F-β-ddA 32	–	36.7	40.0	> 50	> 250	> 50	> 6.8
42	3-Me	3.57	3.57	> 50	91.9	> 50	26
F-β-ddI 41	–	41.7	30.0	> 50	> 250	> 50	> 8
40	3-Me	11.7	12.5	2.83	118	> 50	10
F-β-ddA 33	–	> 250	> 250	> 50	> 250	> 50	n.a.
44	3-Me	75.0	31.7	> 50	> 250	> 50	> 8
F-β-ddI 43	–	> 250	> 250	> 50	> 250	> 50	n.a.
ddA 6	–	4.33	4.55	39.1	> 250	> 50	> 57
d41	–	0.18	0.26	n.d.	56	n.d.	311

^a 50% effective concentration;
^b 50% cytotoxic concentration;
^c Minimal inhibitory concentration;
^d Selectivity index;
n.d.: not determined; n.a.: not available.

amination to d4AMP seems to be the limiting metabolisation step. This supports the assumption that the metabolic profile elaborated for ddA **6** may also be valid for d4A **31**.

The same study has been done using the two 2'-fluorinated ddA derivatives F-β-ddA **32** and F-α-ddA **33**.^{77,86} The only difference is the inverted configuration at carbon C2'. Although at the first glance this may appear only a small difference, it has tremendous impact on the biological activity of these two ddA derivatives. F-β-ddA **33** is biologically active against both HIV-1 and HIV-2 in CEM/O (36 μM), ATH8 (5 μM), and MT-4 cells (35 μM), while F-α-ddA **33** proved completely inactive in the same cell lines (Table 7; Figure 17).^{87,88} Due to its high activity⁸⁹ and particularly due to the complete acid stability⁹⁰ F-β-ddA has been evaluated in clinical trials as an anti-HIV agent. However,

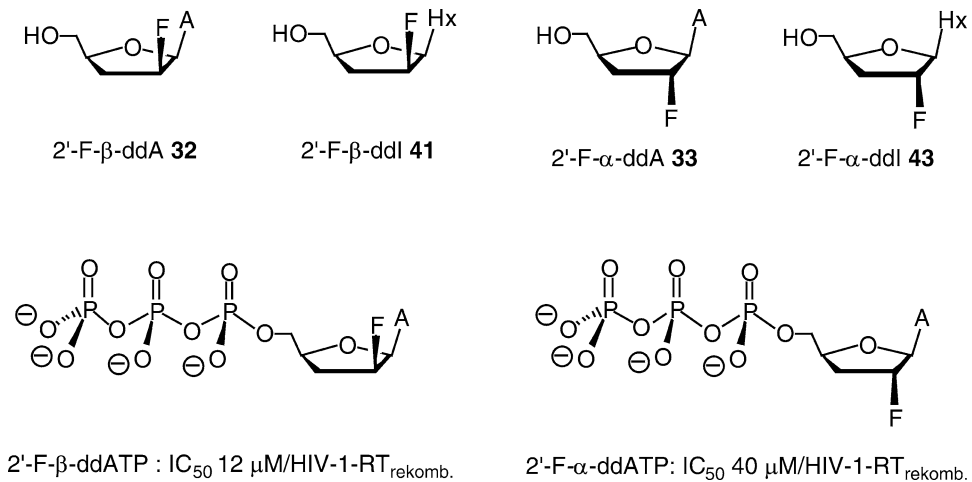


Figure 17. Structures of the studied fluorinated ddA derivatives and the triphosphates.

the trials have been stopped due to toxicity problems. Moreover, both compounds are inactive against Moloney murine sarcoma virus (MSV). However, when the independently synthesized triphosphates of both F-ddA nucleosides were tested for their inhibitory potency against recombinant reverse transcriptase, both triphosphates showed inhibition parameters of IC_{50} 12 μ M (F- β -ddATP) and 40 μ M (F- α -ddATP) (Figure 17).⁹¹ Consequently, at the triphosphate level both compounds are inhibitory to RT (with only a 4-fold difference), while the nucleosides showed a completely different activity. Therefore, the question arose whether it would be possible to locate the metabolism block in the case of F- α -ddA.

The chemical synthesis was carried out as before. Hydrolysis studies revealed again a selective delivery of the corresponding nucleotides. ADA and AMPDA incubations proved the complete stability of the adenine heterocycle. Therefore, again the corresponding fluorinated ddI phosphate triesters were included for comparison.⁷⁷

Interestingly, the antiviral evaluation of 3-methyl-*cyclo*Sal-F- β -ddAMP triester **39** showed a 10-fold increase in bioactivity as compared to the parent nucleoside F- β -ddA **32** (37 μ M), making these compounds as active as ddA **6** (Table 7). 3-Methyl-*cyclo*Sal-F- β -ddIMP **42** was equipotent (3.6 μ M) to 3-methyl-*cyclo*Sal-F- β -ddAMP **39**, which proves that, in contrast to the situation of the ddA(I)/d4A(I) triesters **34–38**, it is unimportant where to enter the metabolic pathway; the re-amination seems not to be the limiting process. F- β -ddI **41** (42 μ M) was equipotent to F- β -ddA **32** (Figure 18). Obviously, for F- β -ddA **32** some limitations during the direct phosphorylation (by deoxycytidine kinase or adenosine kinase), or during the deamination/re-amination steps, may be alleviated by using the *cyclo*Sal approach, thus resulting in an improved antiviral activity. An important difference between 3-methyl-*cyclo*Sal-F- β -ddAMP **39** and 3-methyl-*cyclo*Sal-ddAMP **34** is that with the former only a 10-fold increase in potency is observed relative to the parent drug, whereas with the latter the increase in potency is 100-fold. This may indicate that the activating pathways for the parent compounds is more efficient for F- β -ddA **32** than for ddA **6**, or ddI **7**.⁷⁷ However, due to the considerably lower IC_{50} of ddATP for RT (0.11 μ M)⁹² as compared to F- β -ddATP (12 μ M)⁹¹ the inefficient metabolic activation of the ddAMP prodrug is, in part, compensated. Nevertheless, since there is considerably more ddA loss through catabolic pathways, ddA seems to benefit more than F- β -ddA from the *cyclo*Sal approach.

More striking were the results of 3-methyl-*cyclo*Sal-F- α -ddAMP **40**.⁷⁷ In contrast to the parent nucleoside, 3-methyl-*cyclo*Sal-F- α -ddAMP **40**

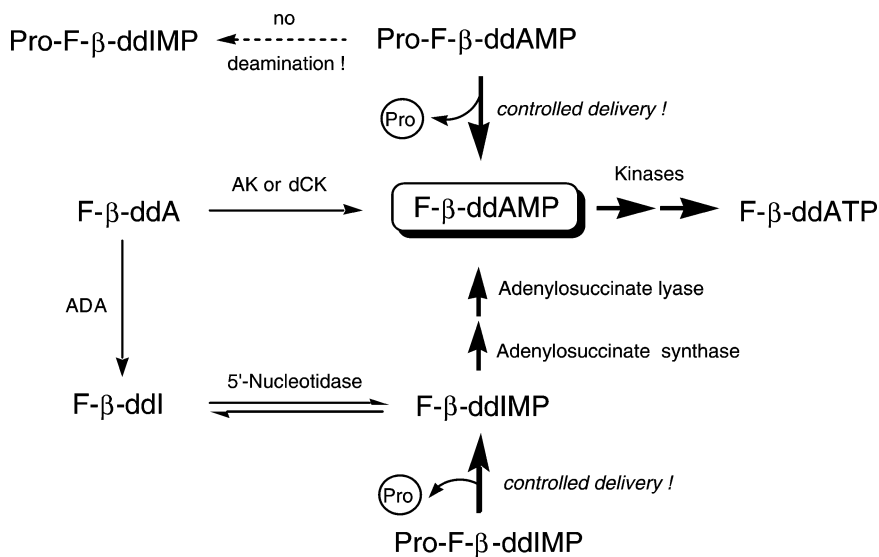


Figure 18. Effect of the use of 3-methyl-*cycloSal*-F-β-ddAMP **39** and 3-methyl-*cycloSal*-F-β-ddIMP **42**.

proved antivirally active at 12 μM (Table 7). This EC₅₀ value is still 3-fold lower as compared to F-β-ddA **32** which showed an EC₅₀ of 37 μM. It is interesting to point out that the difference in antiviral activity of 3-methyl-*cycloSal*-F-β-ddAMP **39** and 3-methyl-*cycloSal*-F-α-ddAMP **40** was 4-fold. So, exactly the same difference that has been found for the corresponding triphosphates against isolated RT (Figure 11).⁹¹ As F-α-ddA **33**, F-α-ddI **43** proved to be completely inactive in the antiviral assay. However, 3-methyl-*cycloSal*-F-α-ddIMP **44** still showed some activity (32 μM against HIV-1). This EC₅₀ value is three-fold higher as compared to the corresponding activity of 3-methyl-*cycloSal*-F-α-ddAMP **40**, which again points to some metabolic problems during the re-amination reactions (Figure 19).

Finally, it should be added that after *cycloSal*-masking both parent nucleosides showed also antiviral activity against MSV in C3H/3T3 cells (1.7–2.8 μM).

Summarizing, both nucleotides F-α-ddAMP and F-α-ddIMP have been converted into bioactive compounds by introduction of the *cycloSal*-masking group. As a consequence, the metabolic blockade has to be located in the metabolism from the nucleoside F-α-ddA **33** to the monophosphate F-α-ddIMP. In our ADA-studies F-α-ddA proved to be the best substrate of all the adenosine analogues discussed in this

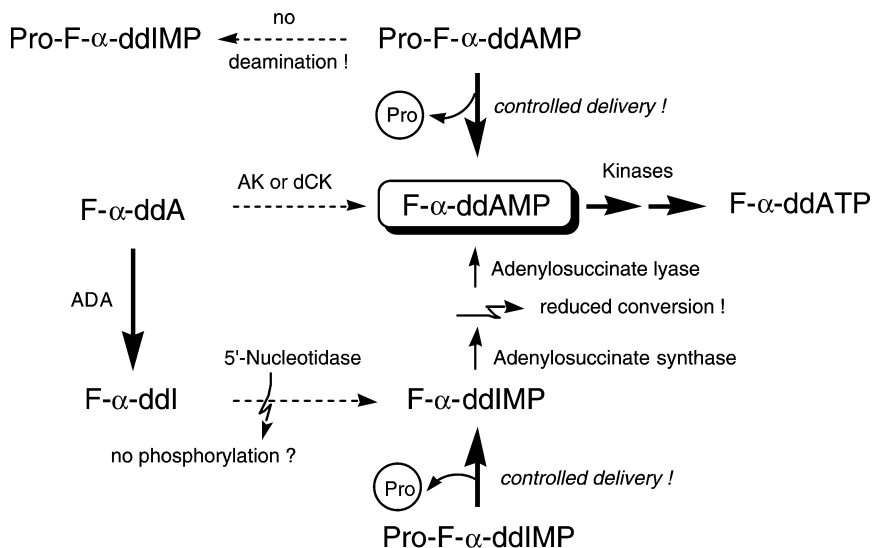


Figure 19. Effect of the use of 3-methyl-*cyclo*Sal-F-α-ddAMP **40** and 3-methyl-*cyclo*-Sal-F-α-ddIMP **44**.

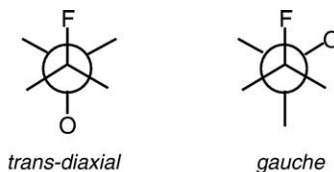
part and it will be very efficiently deaminated to give F-α-ddI. Consequently, this can obviously not be the limiting step, the blockade seems to be the phosphorylation of F-α-ddI **43** into F-α-ddIMP. However, it cannot be excluded that for some reasons the mentioned cleavage of the glycosidic bond by PNP to yield hypoxanthine and the pyrophosphorylated glycon may be a much more efficient degradation pathway for F-α-ddI as compared to the other inosine derivatives. This would lead to a fast clearance of the intracellular F-α-ddI amounts and forward phosphorylation can not compete.

Our hypothesis for this different behavior of the two differently configured F-ddAs should be a specific intrinsic property of these nucleosides. One possible explanation might be that the different stereochemistry of the fluorine substituent is responsible for such striking biological differences between the two compounds because the configuration at C2' is associated with a different conformational effect on the glycon.⁹³ In solution, nucleoside analogues generally show a rapid equilibrium between two equally populated puckering conformations of the glycon: a 3'-endo-(North[N]) conformation and the 2'-endo-(South[S]) conformation. In contrast, a fluorine atom in a 2',3'-dideoxyribose residue leads to a higher rigidity of the five-membered ring and forces the equilibrium to shift to either extreme (N- or S-form)

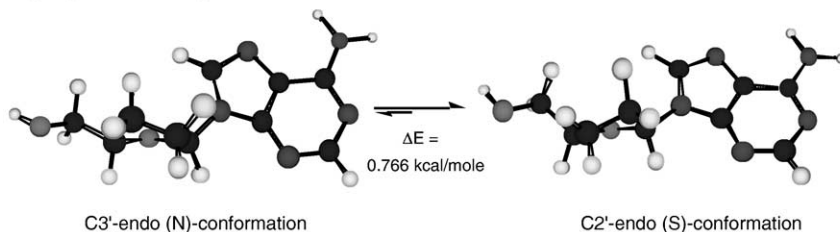
as a function of its stereochemistry.⁹⁴ The origin of the fluorine-induced “stiffness” on the sugar pucker arises from the so-called *gauche*-effect resulting from the interaction between the ribose oxygen and the very electronegative fluorine atom. This strong *gauche*-effect has a profound stereoelectronic effect on the stereochemical orientation of the neighboring groups. Indeed, the fluorine substituent governs the overall conformation of the sugar ring forcing vicinal electronegative atoms to adopt a *gauche*-rotational arrangement instead of the expected trans-diaxial orientation (Figure 12). Due to this *gauche*-effect, the axially-oriented fluorine atom changes the sugar pucker into a preferred 3'-endo-conformation for the F- α -configuration and a preponderant 2'-endo-conformation in the case of the F- β -configuration (Figure 20).⁹³ As a result, the fluorine is able to shift the sugar pucker equilibrium to a high percentage of only one (N- or S-form) conformation of the tetrahydrofuran ring.⁹⁴ In molecular modelling calculations using the MM2 force field we confirmed this effect: the 2'-endo-conformation was found to be 0.766 kcal/mol energetically more favored as the 3'-endo-conformation in F- β -ddA **32**. In contrast, the 3'-endo-conformation was found to be 1.56 kcal/mol more favored as the 2'-endo-conformation in F- α -ddA **33** (Figure 20).

An energy difference of the latter magnitude is sufficient to nearly fix the conformation in only one sugar pucker. These results were confirmed by NMR conformational analyses of the two F-ddA derivatives. As predicted, F- α -ddA was found exclusively in the 3'-endo-conformation, while F- α -ddA showed an equilibrium of about 85:15 of 2'-endo- and 3'-endo-conformation.^{94c} It is important to mention that further modeling and NMR conformational analyses showed that these effects on the sugar pucker of the glycon ring were not further influenced by the attachment of phosphate residues at the 5'-position. The nucleoside monophosphates as well as the nucleoside triphosphates showed exactly identical conformational behavior as the parent nucleosides.⁹¹ Therefore, one possible explanation for the metabolism blockade for F- α -ddA might be that the enzyme 5'-nucleotidase that should convert F- α -ddI into F- α -ddIMP does not accept the 3'-endo-conformation of the sugar pucker in F- α -ddI. Marquez and coworkers⁹⁵ have shown that such conformational effects may indeed play a role in enzymatic conversions. They showed convincingly that adenosine analogues exhibiting a strong preference to a 3'-endo-conformation are clearly better substrates for the deaminating enzyme adenosine deaminase (ADA).⁹⁵ Therefore, F- α -ddA is a much better substrate as compared to F- β -ddA for ADA. A preferred conformation in the glycon ring has also been observed in 2'-fluoro-2',3'-dideoxynucleoside 5'-

the conformational gauche-effect:



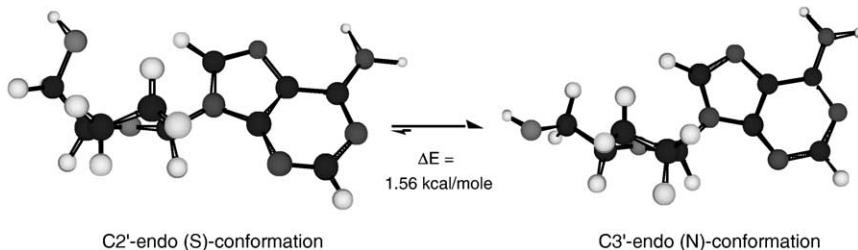
sugar puckers in F- β -ddA **32**



antivirally active nucleoside analogue:

EC₅₀ HIV-1 : 35 μM (MT-4); 5 μM (ATH 8); 36 μM (CEM/O)

sugar puckers in F- α -ddA **33**



antivirally inactive nucleoside analogue (in HIV-1 infected MT-4, ATH 8 and CEM/O)

Figure 20. Conformational preponderance of the fluorinated ddA glycon ring due to the gauche-effect.

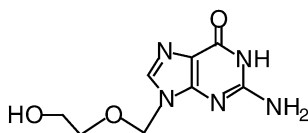
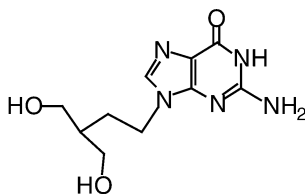
triphosphates interacting with the HIV-1 RT active site.⁹¹ Alternatively, the catabolic enzyme purine nucleoside phosphorylase (PNP) may have the same preference as ADA has. This would lead to an efficient clearance of F- α -ddI due to cleavage of the glycosidic bond and this would prevent the phosphorylation into F- α -ddIMP. If F- β -ddI is less efficiently catabolized it will have a better chance to get phosphorylated into F- β -ddIMP. Further studies have to be performed in order to verify this hypothesis.

Nevertheless, this study shows the great potential of a working pronucleotide system: converting an inactive nucleoside analogue into an antivirally active compound. Due to the intracellular nucleotide delivery at the same time the *cycloSal*-pronucleotide system enables to study biosynthetic pathways which could not be studied before because of the inability to get the nucleotides into the cell. The same has been done before in the case of the *cycloSal*-AZTMP triesters.

IX. *cycloSAL*-PRONUCLEOTIDES AGAINST DNA VIRUSES

DNA viruses are also an attractive target for the application of pronucleotides. In contrast to RNA viruses, these viruses do not rely on reverse transcription of their genome prior to replication. Therefore, the target is not reverse transcriptase but a viral DNA-polymerase.⁹⁶ Moreover, some of the known antivirals against DNA viruses are not monophosphorylated by cellular thymidine kinase but by a viral-encoded thymidine kinase that may have associated thymidylate kinase activity as in the case of HSV-1 and VZV. This leads directly to the formation of the corresponding diphosphates and to a specific activation of the nucleoside analogue only in cells where the virus is present. This has a tremendous advantage for the selectivity of these compounds against TK⁺ HSV and VZV but the disadvantage of not being activated if the virus-encoded TK is absent or deficient.⁹⁷ Exactly this seems to be the case if the virus develops resistance to the drug.⁹⁸ However, not all the virus types belonging to the herpesvirus family express viral thymidine kinase activity, e.g., cytomegalovirus (CMV) does not, and whether Epstein-Barr virus (EBV) would do so is unclear.

Among the most active and most broadly applicable nucleoside antivirals in this area is the purine bearing acyclic nucleoside analogue acyclovir (ACV **45**, Figure 21).⁹⁹ ACV is a potent inhibitor of herpes simplex virus type-1 (HSV-1), herpes simplex virus type-2 (HSV-2), but to a lesser extent varicella-zoster virus (VZV) and CMV and EBV. Acyclovir acts in its triphosphate form as a chain terminator (because its incorporation into DNA does not allow further chain elongation) and/or act as inhibitor of the HSV DNA polymerase (K_i value for ACVTP *versus* the natural substrate dGTP of 0.1 μ M). Although ACV is preferentially phosphorylated by a viral thymidine kinase, it is not a particularly good substrate for HSV-TK and the rate of phosphorylation is considered to be slow. Furthermore, ACVTP has a relatively short intracellular half-life of 0.7 h. More important, resistance to acyclovir

Acyclovir **45** (ACV)HSV-1 : $EC_{50} = 3.8 \mu\text{M}$ (Vero cells)HSV-2 : $EC_{50} = 2.5 \mu\text{M}$ (Vero cells)also active against VZ-Virus ($EC_{50} = 3.5 \mu\text{M}$)
CM-Virus and EB-VirusPenciclovir **46** (PCV)HSV-1 / 2 : $EC_{50} = 6.4 \mu\text{M}$ (Vero cells)also active against VZ-Virus ($EC_{50} = 3.1 \mu\text{M}$)
and EB-Virus**Figure 21.** Acyclic nucleoside analogues with biological activity against DNA viruses.

seems to be associated with a down-regulation of the expression of viral thymidine kinase. The most common mutation is the selection of mutants deficient in TK activity or in mutants that express TKs with altered substrate specificity.

A second acyclic nucleoside analogue that should be mentioned here is penciclovir (PCV, Figure 21).¹⁰⁰ As ACV it is active against HSV-1/2, VZV, CMV, and EBV. Compared to ACV, the improved potency of PCV is attributed to the increased half-life of the major metabolite PCVTP. One major hurdle in the use of PCV is its low bioavailability. The mode of action of PCV has been summarized recently.¹⁰¹

The chemical synthesis of the 3-methyl-*cyclo*Sal-ACVMP triester **47** may be carried out using the chlorophosphite method.¹⁰² However, much better results were obtained when the exocyclic amino group of the guanine residue was protected by dimethoxytritylation prior to the phosphorylation reaction using the phosphoramidite strategy using pyridinium chloride as activating agent. Reproducible yields of 80% were obtained.¹⁰³ The protecting group has been cleaved off by acid treatment of the N²-blocked *cyclo*Sal-triester. The *cyclo*Sal-ACVMP triester **47** was obtained as a racemic mixture. Chemical hydrolysis showed again selective delivery of ACVMP.

Antiviral evaluation showed an EC_{50} for the parent nucleoside ACV **45** of $0.62 \mu\text{M}$ against HSV-1/TK⁺ (Kupka strain; Vero cells). As expected, ACV lost its activity in Vero cells infected with HSV-1/TK⁻ (B2006 strain; $EC_{50} 58 \mu\text{M}$). Strikingly, 3-methyl-*cyclo*Sal-ACVMP **47** showed antiviral activity values of 0.47 and $0.51 \mu\text{M}$ in the same systems, respectively, and an EC_{90} of $1.62 \mu\text{M}$ against the mutant virus strain without increasing the toxicity (Table 8). Again, this complete retention of activity clearly proves that ACVMP is delivered to the cells

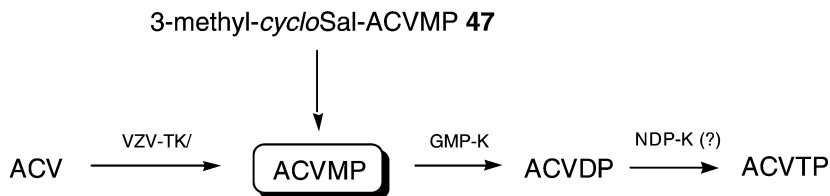
Table 8. Anti-Herpes Virus Activity of 3-Methyl-*cyclo*Sal-ACVMP **47**, *cyclo*Sal-PCVMPs **48a,b**, and *cyclo*Sal-BVDUMP Triesters **51a–d**

Comp.	Subst. X	Antiviral activity EC ₅₀ (μM) ^a								
		HSV-1/TK ⁺ Kupka ^c	HSV-1/TK [−] B2006 ^c	HSV-1/TK ^{−b} B2006 ^c	VZV/TK ⁺		VZV/ TK [−]		CMV	
					YS ^c	OKA ^c	07/1 ^d	YS/R ^d	AD-169 ^d	Davis ^d
47	3-Me	0.47	0.51	1.62	4.1	1.2	7.9	7.6	13	9
ACV 45	–	0.62	57.7	220	5.2	2.6	111	191	> 200	> 200
48a	3-Me/3'-OH	92	108	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
48b	3-Me/3'-OAc	111	119	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PCV 46	–	1.15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
51a	3-Me/3'-OAc	n.d.	n.d.	n.d.	0.069	0.023	> 50	> 5	n.d.	n.d.
51c	3-Me/3'-OH	n.d.	n.d.	n.d.	0.028	0.010	> 50	> 50	n.d.	n.d.
52d	3-Me/3'-OPiv	n.d.	n.d.	n.d.	0.098	0.034	31	31	n.d.	n.d.
56	3-Me/3'-OMe	n.d.	n.d.	n.d.	> 50	50	> 50	> 50	n.d.	n.d.
BVDU 50	–	n.d.	n.d.	n.d.	0.033	0.010	> 200	> 200	n.d.	n.d.

^aEffective concentration;^bEC₉₀ (μM);^cVero cells;^dHuman embryonic lung (HEL) cells;

n.d.: not determined.

against VZV/TK⁺



against VZV/TK⁻

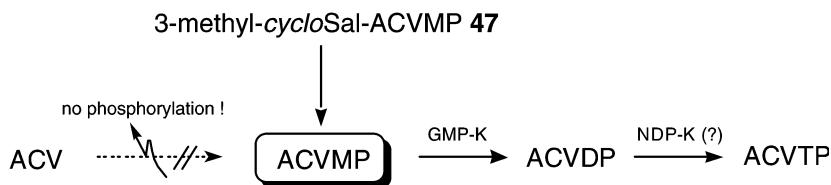


Figure 22. Metabolism of 3-methyl-*cyclo*Sal-ACVMP **47** in VZV/TK⁺ and VZV/TK⁻-infected cells.

by the pronucleotide. Additionally, this also proves that the conversion of ACVMP into ACVDP obviously not depends on the viral thymidylate kinase associated with HSV-thymidine kinase.¹⁰²

A comparable result was obtained for the antiviral activity against VZV.¹⁰⁴ 3-Methyl-*cyclo*Sal-ACVMP **47** showed antiviral activity of EC₅₀ 4.1 and 1.2 μ M against wild-type virus (YS and OKA strain, respectively) (plaque reduction assay). This activity was completely retained in cells infected with VZV/TK⁻ (YS/R strain; 7.6 μ M, Table 8). As expected ACV showed an EC₅₀ of 5.2 μ M against the wild-type virus (YS strain) and an EC₅₀ of 191 μ M against the mutant virus (YS/R strain). As above, viral thymidylate kinase clearly is not involved in the activation into ACVDP. Here, cellular guanosine monophosphate kinase (GMP-K) is responsible for the phosphorylation (Figure 22).

The situation was somewhat different when 3-methyl-*cyclo*Sal-ACVMP **47** was tested against CMV.¹⁰⁴ While ACV itself was completely inactive against two virus strains (EC₅₀ > 200 μ M against the AD-169 and the Davis strain), the triester exhibited activity against *both* virus strain at EC₅₀ 13 and 9 μ M, respectively, without changing the cell morphology (MCC > 200 μ M). This example clearly shows again the potential of the *cyclo*Sal-phosphate triester concept or—in more general—the potential of a pronucleotide: it converts a nucleo-

side analogue which has no antiviral activity against a certain virus into an bioactive compound.

Finally, *cycloSal*-triester **47** proved to be a potent and selective inhibitor of EBV DNA synthesis and EB-virus capsid antigen expression.¹⁰⁵

A further evidence for an effective intracellular delivery of ACVMP from the triester could be taken from the observed genotoxic effects caused by 3-methyl-ACVMP **47** in wild-type CHO cells. The triester caused sister-chromatid-exchange (SCE) and aberrations in this cell line in a concentration dependent manner. Quantitatively comparable rates were found for ACV itself in CHO/TK⁺ gene-transfected CHO cells.¹⁰⁶ This clearly proves that ACVMP was liberated in the former case.

Interestingly, the *cycloSal*-penciclovir phosphate triesters **48a,b** showed a completely different behavior.¹⁰⁵ Two types of PCVMP pronucleotides have been prepared:

- (I) 3-methyl-*cycloSal*-PCVMP **48a** with an unmodified 3'-hydroxyl group and
- (II) 3-methyl-*cycloSal*-(3'-*O*-acetyl)-PCVMP **48b**.

Both compounds were prepared using a common synthetic pathway using appropriately protected PCV precursors where phosphitylation was achieved by the chlorophosphite/oxidation route. Finally, the 3'-hydroxyl group was acetylated.

Biological evaluation (Table 8) revealed a complete loss of the activity against HSV-1/TK⁺ (Kupka strain) of both compounds (EC₅₀ 90 and 111 μ M, respectively). The parent nucleoside PCV **46** was active at 1.15 μ M. A comparable loss in antiviral activity was also found in the EBV DNA synthesis assay and the EB-VCA expression assay. PCV was active at 3.2 and 6.0 μ M, while triester **48a** was "active" at 21 and 35 μ M, respectively. This result was completely against our expectation. However, in chemical hydrolysis studies we observed that not PCVMP but cyclic penciclovir monophosphate **49** was formed from the triester (Figure 23).¹⁰⁷ The structure of cPCVMP **49** has been identified unambiguously by ESI-mass spectrometry in the hydrolysis mixture. So, in contrast to the *cycloSal*-triesters of FdUMP where no formation of the corresponding cFdUMP was observed, the intramolecular nucleophilic attack of the hydroxyl group at the phosphorus center is clearly favored over the intermolecular reaction that would lead to PCVMP.

Moreover, the *cycloSal*-PCVMP triesters were completely inactive to induce genotoxic effects like SCE or aberrations which have already

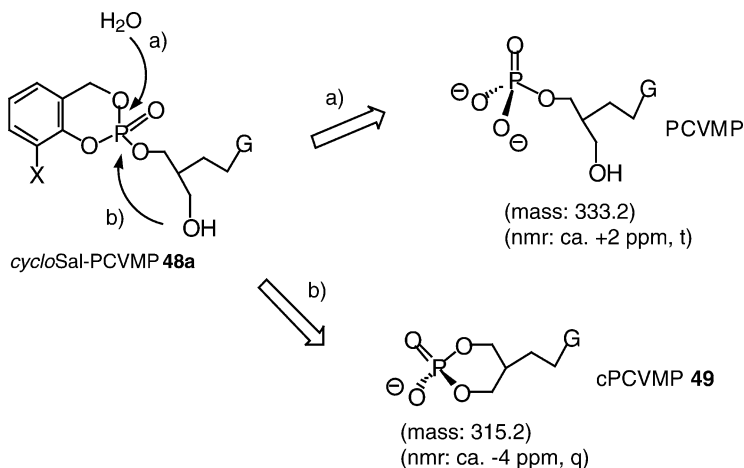
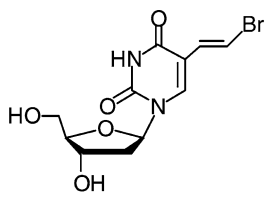


Figure 23. Formation of cyclic penciclovir monophosphate **49** (cPCVMP) by an intramolecular nucleophilic reaction.

been observed for the corresponding *cycloSal*-ACVMP triesters in CHO cells.¹⁰⁶ Again, this proves that no PCVMP is delivered from the triester. In addition, the acyclic guanine analogue ganciclovir (GCV) was also masked by the *cycloSal* method to yield the corresponding 3-methyl-*cycloSal*-GCVMP. The difference of the two nucleoside analogues is only a substitution of a methylene group in the backbone of PCV by an oxygen atom in GCV. Therefore, also a comparable behavior for this compound leading to the formation of cGCVMP was expected. Quite intriguing, the compound proved to be about 100-fold more genotoxic as the parent nucleoside analogue GCV in wild-type CHO cells but still 10-fold less genotoxic than GCV in HSV/TK⁺ gene transfected CHO cells.¹⁰⁶ These results clearly point to an efficient intracellular delivery of GCVMP again. The reason why the replacement of the methylene fragment by an oxygen did not lead to the formation of the cyclophosphate cGCVMP remains unclear and is currently a subject of further study.

That an unusual reaction took place has already been deduced from the observation that in contrast to all 3-methyl-*cycloSal*-triesters studied before the half-life of 3-methyl-*cycloSal*-PCVMP **48a** in chemical hydrolysis studies was considerably reduced (1.6 h *versus* about 15–20 h for other 3-methyl-*cycloSal*-triesters). This unexpected behavior may be attributed to the higher flexibility of the acyclic backbone of PCV as compared to the more rigid glycon structure in FdU. In principle this preferred intramolecular reaction cannot take place in



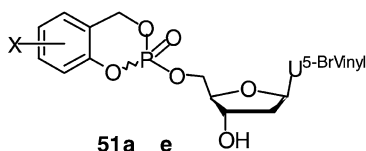
BVDU (Brivudin)

Active nucleoside analogue against Herpes-viruses:

active against HSV-1 but not HSV-2

highly active against VZV (shingles and chickenpox)

not active against EBV ("students disease")



51a e

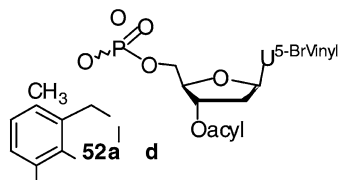
a : X = H

b : X = 5-OMe

c : X = 3-Me

d : X = 3,5-DiMe

e : X = 3-tBu



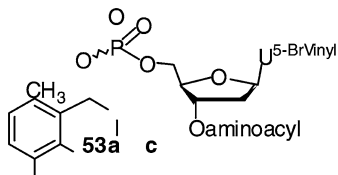
52a d

a : acyl = acetyl

b : acyl = propionyl

c : acyl = hexanoyl

d : acyl = pivaloyl



53a c

a : aminoacyl = alaninyl

b : aminoacyl = phenylalaninyl

c : aminoacyl = prolinyl

Figure 24. Structure of Brivudin (BVDU **50**) and the *cycloSal*-BVDUMP triesters **51–53**.

3-methyl-*cycloSal*-(3'-*O*-acetyl)-PCVMP **48b** but in case of a fast enzymatic deesterification of the acetyl ester group 3-methyl-*cycloSal*-PCVMP **48a** is formed again. That the 3'-hydroxy group plays indeed a crucial role can be seen from the half-life of 3-methyl-*cycloSal*-(3'-*O*-acetyl)-PCVMP **48b** against chemical hydrolysis: $t_{1/2}$ was now found to be again 14 h.

Finally, the *cycloSal* concept has been applied to the nucleoside analogue 5-[(*E*)-2-bromovinyl]-2'-deoxyuridine (BVDU or Brivudin **50**, Figure 24),¹⁰⁸ which is a potent and highly selective inhibitor of the replication of HSV-1 and particularly VZV.¹⁰⁸ Interestingly, BVDU is not markedly active against HSV-2 or EBV.¹⁰⁹ Again, selectivity as inhibitor primarily depends upon a specific activation by HSV-encoded thymidine-kinase (TK) to the mono- and diphosphate and finally to

the triphosphate by cellular enzymes. BVDU-triphosphate (BVDUTP) can act either as an inhibitor of the cellular DNA polymerase or as an alternate substrate that would render the DNA more prone to degradation when incorporated into DNA.¹¹⁰ Some limitations for the use of BVDU are known, i.e., BVDU will be enzymatically degraded to the nucleobase 5-[(*E*)-2-bromovinyl]uracil within 2–3 h.¹¹⁰ Moreover, it has been shown that EBV does not express an HSV-like thymidine kinase and this may be the reason why BVDU is inactive to inhibit EBV-replication,¹¹¹ and, obviously, cytosolic TK is unable to activate BVDU. Our aim was to prove if the *cycloSal* concept is able to broaden the application of BVDU against Epstein–Barr-virus (EBV) infections.¹¹² These play a significant role as secondary infection in, e.g., AIDS patients. It should be mentioned that two attempts at designing pronucleotides of BVDU have been published before but both were unsuccessful.¹¹³ We synthesized differently substituted *cycloSal*-BVDUMP **51** and a series of 3'-*O*-modified derivatives **52** and **53**.¹¹⁴ For 3'-*O*-modification different lipophilic carboxylic acids (**52**) as well as α -amino acids (**53**) were used (Figure 24).

The compounds were prepared using 3'-*O*-levulinylated BVDU or 3'-*O*-esterified BVDU derived from *N*-Boc- α -aminoacids like L-/D-alanine, L-phenylalanine as well as carboxylic acids. These compounds were phosphorylated using the phosphoramidite/oxidation method. The levulinyl protection group was removed by treatment of the triester with hydrazine hydrate. The *N*-Boc protecting group was removed by treatment with trifluoroacetic acid.^{114a}

First, chemical hydrolysis studies proved clearly the selective delivery of BVDUMP as the sole product without formation of 3',5'-cyclicBVDUMP **54** (Figure 25). The half-lives were in the expected order of magnitude. *CycloSal*-BVDUMP triesters modified by esterification with a carboxylic acid (**52**) led to the formation of the 3'-esterified BVDUMP **55** with comparable half-lives. However, the 3'-aminoacyl-esterified compounds **53** showed shorter half-lives and to our surprise the main product was 3-methyl-*cycloSal*-BVDUMP **51c** again and not the corresponding 3'-aminoacyl-esterified BVDUMP **55**. The prototype triester 3-methyl-*cycloSal*-BVDUMP then hydrolysed to yield BVDUMP.

3-Methyl-*cycloSal*-BVDUMP **51c** as well as the 3'-*O*-acetyl derivative **52a** were tested against inhibition of VZV replication.¹⁰⁴ BVDU proved to be highly active against VZV/TK⁺ with EC₅₀ values of 0.033 and 0.010 μ M using the YS and the OKA strain, respectively (Table 8). As expected, this activity was completely lost when VZV/TK⁻ (YS/R and O7/1 strain; > 200 μ M) was used. Interestingly, two 3-methyl-

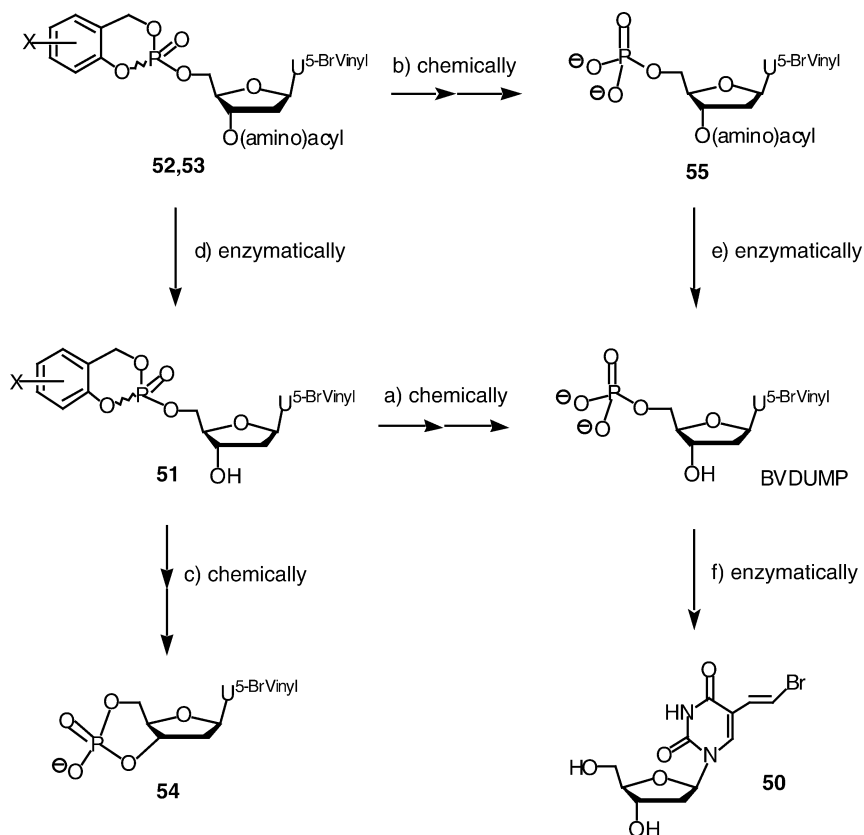


Figure 25. Degradation pathways of the prototype *cycloSal*-BVDUMPs **51** and the 3'-modified derivatives **52**, **53**.

cycloSal-BVDUMP triesters **51c**, **52a** showed comparable anti-VZV activity as compared to the parent BVDU. However, both of them also lost all antiviral activity against the VZV/TK⁻ strains ($EC_{50} > 50 \mu M$, Table 8). As expected, 3'-*O*-methylated 3-methyl-*cycloSal* triester proved to inactive against VZV. Obviously, the intracellular delivery of BVDUMP does not seem to guarantee activity as against the wild-type virus strains. This leads to the conclusion that only the VZV/TK associated viral thymidylate kinase activity is responsible for the formation of BVDUDP intracellularly. There seems to be no cellular enzyme present to phosphorylate BVDUMP as in the case of ACVMP (Figure 26).

^bCC₅₀: concentration required to reduce the growth of exponentially growing P3HR-1 cells by 50%.

Much more interesting were the results obtained in the assays against inhibition of EBV replication in P3HR1 cells.^{114a,b} Here, BVDU was completely inactive ($EC_{50} > 100 \mu\text{M}$ in the EBV DNA synthesis assay as well as the EB-VCA expression assay).¹¹¹ Strikingly, some of the *cycloSal*-BVDUMP triesters exhibited pronounced anti-EBV activity. The most active compound was the prototype 3-methyl-*cycloSal*-BVDUMP **51c**. As compared to the completely inactive BVDU **50**, triester **51b** was > 166-fold more active and about 4-fold more active than the reference compound acyclovir (ACV). The 3'-alanine *cycloSal*-triesters showed antiviral activity that was 5- (D-**53a**) and 12-fold (L-**53a**) lower as compared to **51b**, but both were still significantly more potent than **50**. Surprisingly, all derivatives bearing carboxylic acids (**52a–d**) were devoid of any antiviral activity (Table 9).

Because EBV does not encode a HSV-1-like thymidine kinase (and hence no associated thymidylate kinase), these results clearly indicate that also cellular kinases are unable to activate BVDU to its monophosphate BVDUMP. Releasing BVDUMP from the *cycloSal*-pronucleotide led to the generation of antiviral activity and so the forward phosphorylation of BVDUMP to the ultimate metabolite BVDUTP has to be done by cellular kinases.

This again suggests that *cycloSal*-triesters may be used as biochemical tool to study nucleoside metabolism as shown before for the AZT triesters, the fluorinated ddA triesters as well as acyclovir triesters directed against CMV.

In order to understand the different behavior of the mentioned 3-methyl-*cycloSal*-BVDUMP triesters several studies using isolated carboxyesterase^{114b} and particularly cell extracts were performed. Cell extracts from CEM/O cells^{114d} as well as P3HR1 cells^{114a} (that were used for the antiviral evaluation) were used.

Studies concerning a possible enzymatic contributions caused by intracellular (carboxy)esterases, the triesters **52a–d** and D/L-**53a**, L-**53b** were treated in phosphate buffer, pH 7.3 with 50 units of pig liver esterase (PLE) as a model for contributing enzymes. The half-lives were found to be considerably lower as compared to the studies in phosphate buffer at the same pH but without PLE (Table 10). More importantly, the product was in all cases the 3'-*O*-deesterified prototype triester 3-methyl-*cycloSal*-BVDUMP **51c**, which points to an efficient enzymatically driven process that also explains the shorter half-lives found in the study. After enzymatic deesterification, 3-methyl-*cycloSal*-BVDUMP yielded selectively BVDUMP as in the chemically driven hydrolysis studies. In all incubation mixtures we were unable to detect the co-formation of 3'-*O*-esterified BVDUMP **55**. It is worth mention-

Table 10. Incubation Studies of the *cyclo*Sal-BVDUMP Triesters **51–53** with Pig Liver Esterase (PLE) and in CEM and P3HR1 Cell Extracts

Comp.	Incub. with PLE	Hydrolysis in cell extracts ^a <i>t</i> _{1/2} (h) and [products (%)]	
		CEM cell extract	P3HR1 cell extract
51a	n.a.	2.3 [BVDUMP 82, 50 18]	2.3 [BVDUMP 51, 50 49]
51b	n.a.	3.0 [BVDUMP 58, 50 10]	3.2 [BVDUMP 42, 50 24]
51c	n.a.	4.8 [BVDUMP 50, 50 8]	8.9 [BVDUMP 31, 50 18]
51d	n.a.	9.8 [BVDUMP 38, 50 10]	10.9 [BVDUMP 22, 50 15]
51e	n.a.	38 [BVDUMP 9, 50 < 1]	30 [BVDUMP 9, 50 < 1]
52a	3.6 [51c]	4.1 [BVDUMP 22, 51c 24, 55a 17, 50 4]	2.2 [BVDUMP 16, 51c 29, 55a 16, 50 10]
52b	2.8 [51c]	4.7 [BVDUMP 3, 51c 3, 55b 18, 50 < 1]	4.5 [BVDUMP 3, 51c 4, 55b 13, 50 2]
52c	2.1 [51c]	19 [BVDUMP 5, 51c 3, 55c 2, 50 < 1]	7.7 [BVDUMP 7, 51c 8, 55c 3, 50 5]
52d	2.8 [51c]	11 [BVDUMP 1, 51c 2, 55d 10, 50 < 1]	7.0 [BVDUMP 1, 51c 2, 55d 14, 50 < 1]
D- 53a	0.3 [51c]	0.8 [BVDUMP 52, 51c 38, 50 10]	0.5 [BVDUMP 33, 51c 43, 50 24]
L- 53a	0.6 [51c]	0.08 [BVDUMP 53, 51c 37, 50 10]	0.12 [BVDUMP 30, 51c 44, 50 26]
D- 53b	n.d.	n.d. [BVDUMP 53, 51c 35, 50 12]	n.d. [BVDUMP 35, 51c 40, 50 25]
L- 53b	1.2 [51c]	0.2 [BVDUMP 47, 51c 39, 50 14]	1.1 [BVDUMP 29, 51c 46, 50 25]
D- 53c	n.d.	n.d. [BVDUMP 47, 51c 39, 50 14]	n.d. [BVDUMP 30, 51c 42, 50 28]
L- 53c	n.d.	n.d. [BVDUMP 49, 51c 36, 50 15]	n.d. [BVDUMP 29, 51c 44, 50 27]

^a Results shown in the table are at the end of an 8 h-incubation; missing percentage to 100% is remaining *cyclo*Sal-phosphate triester;

n.a.: not available; n.d.: not determined.

ing that the 3'-O- α -amino acid containing triester **53** was cleaved to the same extent as compared to the 3'-O-carboxylic acid bearing derivatives **52**. It seems that PLE has no preference to cleave α -amino acid esters as compared to esters formed from "normal" carboxylic acids (Table 10). As a conclusion from these studies it should be possible to cleave all 3'-O-ester groups readily by enzymatic deesterification as a main degradation pathway to yield the prototype 3'-O-unmodified triester **51c**. Therefore, the reasons for the pronounced differences in antiviral activity remain unclear (Table 9). Moreover, these results clearly show that the use of chemical hydrolysis and PLE-catalyzed deesterification did not provide sufficient data in order to explain the behavior of the compounds in cellular media.

Next, P3HR-1 cell extracts were used. A few striking differences have been observed. The prototype **51c** was degraded to BVDUMP with a half-life of 8.9 h (Table 10). This value is comparable to that found in the chemical hydrolysis studies. Hence, the degradation is purely chemically and not enzymatically driven. No cBVDUMP **54** formation was detected.¹⁰⁷ Besides BVDUMP, BVDU **50** was observed after 4 h of incubation (5%) and after 8 h (22%) to a minor extent, which is due to an enzymatic dephosphorylation of BVDUMP by phosphatases/nucleotidases (path f, Figure 25). In independent studies BVDUMP was converted to an extent of 13% to BVDU within 4 h.

The hydrolyses of the 3'-O-acyl derivatives **52** exhibited a clear difference with respect to the attached acid. For the 3'-O-Ac-derivative **52a** enzymatic deesterification by carboxyesterases yielded the prototype **51c** as the major product (32%) but only 9% of BVDUMP was found. In contrast to that, compounds **52c** (3'-O-Hex) gave only 7% of BVDUMP and 8% of 3-methyl-cycloSal-BVDUMP **51c**. The 3'-O-Prop derivative **52b** and the 3'-O-Piv compound **52d** yielded the 3'-O-esterified BVDUMP derivatives **55c,d** as the major hydrolysis products due to the chemically driven cleavage of the cycloSal-mask (path b) (Table 10). It is noteworthy that for compounds **52a-d** only about 10% conversion into BVDUMP was observed.

The situation was significantly different for the α -amino acid modified 3-methyl-cycloSal-BVDUMP triesters **53a-c**. All six compounds were rapidly deesterified to yield the prototype triester **51c** as major product (40–44%). This is a significant difference as compared to the PLE studies described above. The half-lives were dependent on the stereochemistry and the type of the α -amino acid (Table 10). Moreover, after 8 h incubation a total of 33% BVDUMP was formed starting from both the alanine esters D/L-**53a**. At the same time point 31% BVDUMP was formed starting directly from 3-methyl-cycloSal-BVDUMP **2**.

Comparable data were obtained in the CEM/O cell extract incubation.^{114d} The major difference was that in 8 h incubations the triesters yielded about 50% and about 50% BVDUMP starting from 3-methyl-*cycloSal*-BVDUMP **51c** or 3-methyl-*cycloSal*-3'- α -aminoacyl-BVDUMP **53**, respectively. Again, the triesters modified at C3' with a carboxylic acid exhibited considerable lower amounts of BVDUMP (1–22% in 8 h incubations, Table 10). Moreover, CEM/O cell extracts seem to dephosphorylate BVDUMP to a lower extent as compare to P3HR1 cell extracts (13% vs 26%, respectively).

Taking these data together, the experiments in the cell extracts result in a much higher formation of BVDUMP for the prototype triesters as well as the aminoacid-modified triesters as compared to the carboxylic acid-modified derivatives. Extrapolating to the situation inside the cells, this would lead to considerable BVDUMP amounts and this seems to result in a high degree of forward phosphorylation to the ultimate metabolite BVDUTP. These tests and the antiviral evaluation proved that modification of the 3'-hydroxyl group by simple carboxylic acids abolish all biological activity for unknown reasons while the use of α -amino acids keeps the activity. The advantage of the latter compounds is their higher solubility in aqueous media even as compared to prototype **51**.

The promising antiviral data of some of the reported *cycloSal*-BVDUMP triesters (e.g., **51c**) proved that the *cycloSal*-approach is able to convert the anti-EBV inactive BVDU into a bioactive agent. Finally, to the best of our knowledge the work reported here represents the first example of the application of a pronucleotide approach to a nucleoside analogue possessing a 3'-hydroxyl group with considerable improvement of the antiviral activity.

X. CONCLUSION

Summarizing, the *cycloSal* approach demonstrated convincingly the intracellular delivery of the nucleotide and is the first example of a successful nucleotide delivery system that can be activated by non-enzymatic hydrolysis using a tandem reaction. It has considerably improved the antiviral activity of certain nucleoside analogues. As compared to the bis(SATE) approach^{33,35} or the phosphoramidate approach,⁴⁵ the biological potency is comparable. This is essentially the case when our concept is compared side-by-side with the phosphoramidate approach. The advantages of the *cycloSal* concept are the facile synthetic excess and the reasonable solubility of the compounds

in aqueous media as compared to the other methodologies. Moreover, the drug/masking group ratio in the *cycloSal* concept is 1:1, whereas in almost all nucleotide delivery systems that require enzyme activation the ratio is at least 1:2. Obviously, this 1:1-ratio may be favorable in terms of potential toxicity effects. Additionally, we believe that a pronucleotide that can be cleaved by pH-control should have advantages in *in vivo* applications because this approach is not directly dependent on different enzyme concentrations within a living system.

Although the *cycloSal* approach yielded compounds with superior antiviral activity to the parent nucleoside, there are still requirements with regard to certain aspects of the masking group, to solubility, to transport phenomena, and to selective trigger activation mechanisms that have to be addressed. Yet it has been shown that the *cycloSal*-pronucleotide system is an ideal biochemical tool to study biochemical pathways in nucleoside metabolism.

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