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PREFACE

This represents the fifth volume of *Advances in Antiviral Drug Design*, continuing the tradition of the preceding volumes published by JAI Press, Inc. in 1993 (volume 1), 1996 (volume 2) and 1999 (volume 3), and Elsevier in 2004 (volume 4). Akin to volume 4, the fifth volume of *Advances in Antiviral Drug Design* is also published by Elsevier.

As has been set from the beginning, *Advances in Antiviral Drug Design* is aimed at keeping up with the progress made in the design and development of new antiviral drugs, thereby overviewing the global area where antiviral agents have started to emerge, where they are targeted at, and how they are likely to evolve into clinically useful antiviral medicines.

The principal aim of this series of treatises is to (try to) form a bridge from the basics (i.e., design and synthesis of new antiviral compounds) to clinics (i.e., therapeutic applications in the therapy and/or prophylaxis of well-defined viral infections). This is a continuously evolving process which is driven by a number of forces, the emergence of new viruses (or re-emergence of old ones), the synthesis of new chemical compounds, the molecular optimization of their potency and/or selectivity, and their eventual (medical) application and usefulness in the clinic.

In previous volumes (volumes 1, 2, 3 and 4), the following topics were dealt with:

- (i) uncoating inhibitors for picornavirus infections,
- (ii) broad-spectrum antiviral nucleoside analogs (such as ribavirin),
- (iii) acyclic nucleoside analogs (such as acyclovir and ganciclovir),
- (iv) acyclic nucleoside phosphonates (such as cidofovir and adefovir),
- (v) dideoxynucleoside analogs (such as zidovudine, didanosine, zalcitabine, and stavudine),
- (vi) antisense oligonucleotides,
- (vii) S-adenosylhomocysteine (AdoHcy) hydrolase inhibitors,
- (viii) carbocyclic nucleoside analogs,
- (ix) nucleotide prodrugs (bypassing the initial phosphorylation step),

- (x) HIV protease inhibitors,
- (xi) L-nucleoside analogs (such as 3TC, (-)FTC and L-FMAU),
- (xii) progress with the acyclic nucleoside phosphonates in clinical trials,
- (xiii) emivirine as prototype of the non-nucleoside reverse transcriptase inhibitors (NNRTIs),
- (xiv) zanamivir as prototype of the influenza virus neuraminidase inhibitors,
- (xv) the bicyclams as inhibitors of the replication of T-lymphotropic X4 HIV-1 strains, acting through a specific antagonization of the CXCR4 receptor,
- (xvi) new anti-HIV agents in clinical development,
- (xvii) HIV integrase inhibitors,
- (xviii) non-peptidic HIV protease inhibitors,
- (xix) oseltamivir as prototype of the influenza virus neuraminidase inhibitors,
- (xx) six-membered carbocyclic nucleoside analogues as anti-herpesvirus agents,
- (xxi) cyclosaligenyl pronucleotides of antiviral agents.

In the present volume, volume 5, of *Advances in Antiviral Drug Design*, six new chapters have been added to the list. *First*, an overview of the *status presens* of the antiviral agents which are active against DNA viruses and retroviruses (the latter, being RNA viruses, replicate *via* a proviral DNA intermediate). *Second*, an overview of the *status presens* of the antiviral agents which are active against RNA viruses except retroviruses. In these overview chapters, I have focussed on three categories of antiviral agents:

- (a) those that have been licensed (i.e., approved) for clinical use;
- (b) those that are, or have been, under clinical evaluation (i.e., submitted to phase I, II or III clinical trials); and
- (c) those that are still in the preclinical development stage.

In [Chapter 3](#) J. Zemlicka has addressed a new class of nucleoside analogues, that of the methylenecyclopropane derivatives which yield great potential as antiviral agents against a wide array of herpesviruses.

[Chapter 4](#) written by K.Y. Hostetler is dealing with the synthesis and antiviral activity of orally bioavailable prodrugs of cidofovir and other acyclic nucleoside phosphonates, with broad antiviral drug potential against virtually all DNA viruses.

The HIV co-receptor antagonists, and, in particular, the CCR5 antagonists have proceeded swiftly through clinical development, and this progress has been highlighted by M. Perros in [Chapter 5](#). The non-nucleoside reverse transcriptase inhibitors (NNRTIs) have remained in the frontline of the treatment of HIV-1 infections, and the development of new highly promising congeners of this series, i.e., DATA and DAPY, has been depicted by J. Heeres and P.J. Lewi ([Chapter 6](#)).

Thus, the fifth volume of *Advances in Antiviral Drug Design* aims at continuing with the tradition of the preceding volumes, that is periodically reviewing the recent progress made in the frontline of the design and development of new therapeutic agents for the treatment of DNA virus, retrovirus and RNA virus infections, with, in this volume, particular emphasis on four novel strategies targeted at herpesviruses (i.e., methylenecyclopropane derivatives) and other DNA (*viz.* pox, adeno) viruses (i.e., orally bioavailable prodrugs of acyclic nucleoside phosphonates), and HIV infections (i.e., CCR5 antagonists as well as new highly promising NNRTIs).

E. De Clercq
Editor

STATUS PRESENTS OF ANTIVIRAL DRUGS AND STRATEGIES: PART I: DNA VIRUSES AND RETROVIRUSES

Erik De Clercq

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ABSTRACT

More than 40 compounds have been formally licensed for clinical use as antiviral drugs, and half of these are used for the treatment of HIV infections. The others have been approved for the therapy of herpesvirus (HSV, VZV, CMV), hepadnavirus (HBV), hepacivirus (HCV) and myxovirus (influenza, RSV) infections. New compounds are in clinical development or under preclinical evaluation, and, again, half of these are targeting HIV infections. Yet, quite a number of important viral pathogens (i.e. HPV, HCV, hemorrhagic fever viruses) remain in need of effective and/or improved antiviral therapies.

I. INTRODUCTION

There are at present a forty some antiviral drugs that have been formally licensed for clinical in the treatment of viral infections.⁴⁴ These are mainly used in the treatment of infections caused by human immunodeficiency virus (HIV), hepatitis B virus (HBV), herpesviruses [herpes simplex virus (HSV), varicella-zoster virus (VZV),

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cytomegalovirus (CMV)], orthomyxoviruses (influenza), paramyxoviruses [respiratory syncytial virus (RSV)], and hepaciviruses [hepatitis C virus (HCV)]. As these are the viruses that are most in demand of antiviral therapy, they have prompted the search for new antiviral strategies and drugs directed towards either the same molecular targets as the approved antiviral drugs or to other targets.

Most of the newly described antiviral compounds (that are currently in development) are targeted at HIV, HBV or HCV. Some are targeted at HSV, VZV or CMV, but, there are, in addition, many other important viral pathogens for which medical intervention, either prophylactic or therapeutic, is highly needed, and, these are, among the DNA viruses, the papillomaviruses [human papilloma virus (HPV)], adenoviruses, poxviruses (variola, vaccinia, monkeypox, ...) and the herpesviruses Epstein–Barr (EBV) and human herpesvirus type 6 (HHV-6), and, among the RNA viruses, enteroviruses (i.e. Coxsackie B and Echo), coronaviruses [i.e. severe acute respiratory syndrome (SARS)-associated coronavirus], flaviviruses (i.e. Dengue, Yellow fever) and other RNA viruses associated with hemorrhagic fever [arenaviruses (i.e. Lassa fever), bunyaviruses (i.e. Rift Valley fever, Crimean-Congo fever) and filoviruses (i.e. Ebola and Marburg)].

Here I will describe, for each viral family, (i) which are the antiviral drugs that have been formally approved, (ii) which are the compounds that are under clinical development and thus may be considered as antiviral drug candidates, and (iii) which compounds are in the preclinical stage of development and still have a long route ahead before they could qualify as antiviral drugs ([Table 1](#), [Figure 1](#)). The virus families to be addressed are the following: parvo-, polyoma-, papilloma-, adeno-, herpes-, pox-, picorna-, flavi-, corona-, orthomyxo-, paramyxo-, arena-, bunya-, rhabdo-, filo-, reo-, and retroviruses.

II. PARVOVIRUSES

No significant attempts have been made to develop compounds with potential activity against B19, the only parvovirus that is pathogenic for humans and responsible for erythema infectiosum, the so-called fifth disease, in children.

III. POLYOMAVIRUSES

No antiviral drugs have been formally approved for the treatment of polyomavirus (JC and BK)-associated diseases such as progressive

multifocal leukoencephalopathy (PML) and hemorrhagic cystitis in patients with AIDS. There are, however, anecdotal case reports pointing to the efficacy of cidofovir [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine, HPMPC], which has been licensed under the trademark name Vistide® for the intravenous treatment of CMV retinitis in AIDS patients] in the treatment of polyoma (JC and BK) virus infections, particularly PML, in AIDS patients.³⁹

The *in vitro* activity of various acyclic nucleoside phosphonates, among which HPMPC (cidofovir), against murine and primate polyomaviruses has been well established.¹ Esterification of cidofovir (CDV) with hexadecyloxypropyl (HDP) or octadecyloxyethyl (ODE) groups, as in HDP-CDV or ODE-CDV, respectively, resulted in up to a 3-log decrease of the 50% effective concentration (EC₅₀) and up to 2-log increase of the selectivity index.¹³⁰

IV. PAPILLOMAVIRUSES

As for polyomaviruses, no antivirals have been licensed for the treatment of human papillomavirus (HPV)-associated diseases, including warts (*verruca vulgaris*), condyloma acuminatum, papillomatosis (i.e. recurrent respiratory papillomatosis), and cervical, vulvar, penile and (peri)anal dysplasia (evolving to carcinoma). Various strategies, including surgery and other destructive therapies, antiproliferative agents, and immunotherapies have been used for the treatment of HPV-associated lesions.¹⁴¹ Cidofovir has been used “off label”, with success, in the topical and, occasionally, systemic treatment of HPV-associated papillomatous lesions.³⁹ In many instances, a virtually complete and durable resolution of the lesions was achieved following topical application of cidofovir as a 1% gel or cream.

In addition to cidofovir, other acyclic nucleoside phosphonates, such as cPrPMEDAP [N⁶-cyclopropyl-9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine], are being explored for their potential in the treatment of HPV-associated papillomas and dysplasias.^{39,51} These compounds have been shown to specifically induce apoptosis in HPV-infected cells, which, in turn, may be related to their ability to restore the function of the tumor suppressor proteins p53 and pRb (which are neutralized by the oncoproteins E6 and E7, respectively, in HPV-infected cells).

Recently, biphenylsulfonacetic acid derivatives have been described as inhibitors of HPV E1 helicase-associated ATP hydrolysis.^{60,155} Although these novel ATPase inhibitors can hardly be considered to be

good drug candidates, they may serve as leads for further optimization as potential antiviral agents active against multiple HPV types.¹⁵⁵

As for so many other virus infections (see *supra*), RNA interference (RNAi), based on small interfering RNAs, has been advocated to block HPV infections (HPV16 E6 oncogene expression).^{118,148} This siRNA approach could be particularly useful for silencing HPV oncogenes, as in cervical intraepithelial neoplasia.

V. ADENOVIRUSES

For the treatment of adenovirus infections, which could be quite severe in immunocompromised patients (i.e. allogeneic hematopoietic stem-cell transplant recipients), no antiviral drugs have been officially approved. Anecdotal reports have pointed to the efficacy of cidofovir against adenovirus infections in such patients.³⁹ Among the novel compounds that could be further explored for the treatment of adenovirus infections are (S)-2,4-diamino-6-[3-hydroxy-2-phosphonomethoxy]propoxy]pyrimidine [HPMPO-DAPy],⁵¹ which akin to some “older” compounds like (S)-9-(3-hydroxy-2-phosphonomethoxypropyl)adenine (HPMPA), the N7-substituted acyclic nucleoside 2-amino-7-(1,3-dihydroxy-2-propoxymethyl)purine S-2242, the 2',3'-dideoxynucleosides zalcitabine (ddC) and alovudine (FddT, FLT) have been found to inhibit adenovirus replication *in vitro*.¹¹³

In fact, ddC was also found effective *in vivo*, in a mouse model for adenovirus pneumonia.¹¹¹ Also, ether lipid-ester [hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE)] prodrugs of HPMPA and HPMPA have been designed that inhibit adenovirus replication *in vitro* at significantly lower concentrations than the parent compounds.⁷³

VI. α -HERPESVIRUSES (HSV-1, HSV-2, VZV)

For the treatment of HSV-1, HSV-2 and VZV, a number of compounds have been approved: acyclovir and its oral prodrug valaciclovir; penciclovir and its oral prodrug, famciclovir; idoxuridine, trifluridine and brivudin. Penciclovir, idoxuridine and trifluridine are used topically, primarily in the treatment of herpes labialis (penciclovir) and herpetic keratitis (idoxuridine, trifluridine). Acyclovir can be used orally, intravenously or topically, whereas valaciclovir and famciclovir are administered orally, in the treatment of both HSV and VZV infections. Brivudin (available in some European countries) is used orally for the

treatment of herpes zoster, but is also effective against HSV-1 infections.

While acyclovir (and its oral prodrug valaciclovir) have remained the gold standard for the treatment of HSV and VZV infections, few attempts have been made to bring other anti-HSV (or anti-VZV) agents into the clinic, with the exception of the H2G prodrug, which, since quite a number of years, is still in clinical development for the treatment of herpes zoster.⁴⁶ From acyclovir (ACV) and ganciclovir (GCV) tricyclic derivatives, i.e. tricyclic acyclovir (TACV), and 6-substituted derivatives thereof which have similar (or only slightly decreased) antiviral potency but increased lipophilicity as compared to the parent compounds (ACV and GCV), and, in addition, show interesting fluorescence properties.⁶⁸

Worth considering for clinical development as anti-HSV (and anti-VZV) agents are a number of carbocyclic guanosine analogues, such as A-5021, cyclohexenylguanine, and the methylene cyclopropane syn-guanol.⁴⁹ All these compounds owe their selective antiviral activity to a specific phosphorylation by the HSV- or VZV-encoded thymidine kinase (TK); upon phosphorylation to their triphosphate form, they act as chain terminators in the DNA polymerization reaction. In the (rare) circumstances that HSV or VZV becomes resistant to the acyclic (or carbocyclic) guanosine analogues due to TK deficiency (TK⁻), the pyrophosphate analogue foscarnet could be useful to treat TK⁻ HSV or TK⁻ VZV infections (in immunocompromised patients).

Recently, a second generation of methylene cyclopropane analogues, the 2,2-bishydroxymethyl derivatives, has been synthesized.¹⁶⁶ These compounds may have potential, not only for the treatment of HSV-1, HSV-2 and VZV, but also β -herpes (CMV, HHV-6, HHV-7) and γ -herpes (EBV, HHV-8) infections.⁸⁵ In particular, ZSM-I-62 (Cyclopropavir) has been reported to be very effective in reducing mortality of mice infected with murine CMV.⁸⁴ Of a recently synthesized series of 9-[[3-fluoro-2-(hydroxymethyl)cyclopropylidene]methyl]adenines and guanines, the (Z)-[[trans-(3-fluoro-2-hydroxymethyl)cyclopropylidene]methyl]adenine was quoted as being effective against EBV at an EC₅₀ < 0.03 μ M.¹⁶⁷

New anti-HSV agents targeting the viral helicase–primase complex, the thiazolylphenyl derivatives BILS 179 BS and BAY 57-1293, were recently reported to have *in vivo* efficacy in animal models of HSV-1 and HSV-2 infections.^{30,87} These compounds seem to function by diminishing the affinity of the helicase–primase complex for the HSV DNA. The heterotrimeric helicase–primase complex is composed of the UL5, UL8 and UL52 gene products with DNA helicase DNA-dependent ATPase and DNA primase activity.²⁹ Resistance to

aminothiazolylphenyl-based inhibitors has been shown to arise from single amino acid changes in the UL5 protein.¹⁰³

The antiviral potency of BAY 57-1293 was reported to be superior to all compounds that are currently used to treat HSV infections.¹³ In recent studies, BAY 57-1293 was shown to be more efficacious than famciclovir in the therapy of HSV-1 infections in BALB/C mice.¹⁶ Also, BILS 45BS, which is structurally related to BILS 179 BS, exhibited excellent efficacy in the oral treatment of acyclovir-resistant (ACV^r) HSV-1 infections in nude mice,⁵⁷ highlighting the potential of this class of antiherpetic agents for the treatment of ACV^r HSV disease in humans.

RNA interference (RNAi), as generated by small interfering RNAs (siRNAs), has been recently pursued as a powerful tool to silencing disease,¹³⁸ including viral infections. Palliser et al.¹²³ have shown that vaginal instillation of siRNAs targeting the HSV-2 *UL27* and *UL29* genes (which encode an envelope glycoprotein and a DNA binding protein, respectively) protected mice from a lethal HSV-2 infection; the siRNAs were mixed with lipid so as to ensure their uptake by the cells (vagina and ectocervix). From this study it was concluded that siRNAs may be attractive candidates for application as microbicides to prevent viral infection.¹²³

A new class of anti-VZV compounds are the bicyclic furo (2,3-*d*)pyrimidine nucleoside analogues (BCNAs), represented by Cf 1742 and Cf 1743.¹¹⁰ These compounds are exquisitely active against VZV.⁴⁰ They inhibit the replication of VZV, but not that of other viruses (including HSV), at subnanomolar concentrations, with a selectivity index in excess of 100,000.² Given the extremely high potency and selectivity of the BCNAs they warrant to be further developed towards clinical use, i.e. against herpes zoster.

VII. β -HERPESVIRUSES (CMV, HHV-6, HHV-7)

Five compounds have been licensed to treat CMV infections: ganciclovir, its oral prodrug valganciclovir, foscarnet, cidofovir and fomivirsen. [Foscarnet has also proven efficacious in the treatment of other DNA virus (i.e., hepatitis B) infections.⁷²] With the exception of fomivirsen (an antisense oligonucleotide) which targets the CMV immediate-early mRNA, all other licensed anti-CMV drugs target the viral DNA polymerase. Ganciclovir must first be phosphorylated by the CMV-encoded protein kinase (the UL97 gene product) which is also the principal site for mutations engendering resistance towards this compound. Toxic

side effects (i.e. bone–marrow suppression for ganciclovir, nephrotoxicity for foscarnet and cidofovir) have prompted the search for new inhibitors of CMV.⁴¹

Several benzimidazole ribonucleosides, among which maribavir (previously also known as 1263W94), have been accredited with specific activity against human CMV. Maribavir seems to target the UL97 protein kinase,¹⁵ and, as the UL97 gene product has been shown to account for the release of CMV nucleocapsids from the nucleus,⁹⁰ maribavir may be assumed to target a stage in the viral life cycle that follows viral DNA maturation and packaging. Preclinical pharmacokinetic and toxicological studies have shown that maribavir has a favorable safety profile and excellent oral bioavailability.⁸⁹

Phase I/II dose-escalation trials in HIV-infected men with asymptomatic CMV shedding further indicated that maribavir is rapidly absorbed following oral dosing and reduces CMV titers in semen.⁹³ Maribavir is currently in a prophylaxis study in allogeneic stem cell transplant recipients with results expected in 2006 [to be divulged by ViroPharma Inc., according to Biron¹⁴]. Biron¹⁴ also mentioned two other compounds, i.e. BAY 38-4766 and GW275175X, which entered clinical development but were then not further developed despite a favorable safety profile (BAY 38-4766) or shelved in favor of the advancement of maribavir (GW275175X).

While maribavir is primarily active against CMV, 2-chloro-3-pyridin-3-yl-5,6,7,8-tetrahydroindolizine-1-carboxamide (CMV423) has potent and selective *in vitro* activity against all three human β -herpesviruses, CMV, HHV-6 and HHV-7.^{35,36} As compared to ganciclovir and foscarnet, CMV423 has higher antiviral potency and lower cytotoxicity. It is targeted at an early stage of the viral replication cycle (following viral entry but preceding viral DNA replication), which is regulated by a cellular process that may involve protein tyrosine kinase activity.

Some cellular kinase inhibitors have been found to enhance the antiviral activity of maribavir.²⁶ It may be useful, therefore, to further explore the possibility of therapeutically useful combinations of maribavir and cellular kinase inhibitors such as CMV423. Combination of maribavir with ganciclovir should not be recommended, since maribavir antagonizes the antiviral action of ganciclovir.²⁵

Alkoxyalkyl esters of cidofovir (i.e. HDP-CDV) have been developed that retain the efficacy of the parent compound,¹⁰ without the associated renal toxicity²⁸ and with significantly improved oral bioavailabilities. HDP-CDV (CMX001) is under current development as an oral drug for the treatment of poxvirus infections as well as CMV and other herpesvirus infections.

There is, at present, no standardized antiviral treatment for HHV-6 infections and also their potential clinical indications remain ill-defined. From a comparative study, A-5021, foscarnet, S2242, and cidofovir emerged as the most potent compounds with the highest antiviral selectivity against HHV-6.⁵⁰ The latter three also proved to be the most potent against HHV-7.⁵⁰ In addition to cidofovir, HPMPA and its 3-deaza analogue 3-deaza-HPMPA have also been identified as potent and selective inhibitors of HHV-6 replication.¹¹²

However, the most promising anti-HHV-6 activity was demonstrated by CMV423, a compound that has been shown previously to be highly effective *in vitro* against CMV.¹⁴² The compound exhibited a potency (EC_{50} : 0.02–0.05 μ M) and selectivity ($SI > 2000$) against HHV-6(A), which by far exceeded that of the standard anti-herpesvirus agents acyclovir, ganciclovir, foscarnet and cidofovir.⁴⁷ The *in vitro* antiviral actionprofile of CMV423 is such that it deserves to further explored for its *in vivo* potential in the treatment of CMV and HHV-6(A) infections.

VIII. γ -HERPESVIRUSES (EBV, HHV-8)

Although a number of the aforementioned approved anti-herpetic drugs, such as acyclovir, ganciclovir, brivudin and cidofovir, have proven to be effective against the *in vitro* replication of EBV and HHV-8,⁵⁰ none of these (or any other) antiviral drugs have been formally approved for the treatment of diseases associated with EBV (i.e. mononucleosis infectiosa, B-cell lymphoma, lymphoproliferative syndrome, Burkitt's lymphoma, nasopharyngeal carcinoma) or HHV-8 (Kaposi's sarcoma, primary effusion lymphoma, multicentric Castleman's disease). It would seem appealing to further examine established anti-herpetic drugs, such as cidofovir, and other acyclic nucleoside phosphonates such as HPMPA, or prodrugs thereof, for their potential in the therapy of EBV- and HHV-8-associated malignancies.

Also, new nucleoside analogues, such as the conformationally locked nucleoside analogue *north*-methanocarbothymidine [(*N*)-MCT],¹⁶⁹ which have been previously¹³⁴ shown to block the replication of HSV-1 and HSV-2, should be further explored for their potential in the prevention and treatment of HHV-8-associated malignancies: *in casu*, (*N*)-MCT, which is specifically triphosphorylated in HHV-8-infected cells undergoing lytic replication efficiently blocks HHV-8 DNA replication in these cells.¹⁶⁹ In fact, the antiviral activity spectrum of (*N*)-MCT not only includes γ -herpesviruses (EBV, HHV-8) and α -herpesviruses (HSV-1, HSV-2, VZV) but also poxviruses. (*N*)-MCT would be activated

by the viral thymidine kinase (TK) homologs and inhibit the viral DNA polymerase. The compound has been demonstrated to be effective *in vivo* in reducing the mortality of mice infected with HSV-1 or orthopoxviruses.¹²⁵

IX. POXVIRUSES (VARIOLA, VACCINIA, MONKEYPOX, MOLLUSCUM CONTAGIOSUM, ORF ...)

Thiosemicarbazenes, i.e. isatin- β -thiosemicarbazone and N-methylisatin- β -thiosemicarbazone (marboran or methisazone) were investigated in the 1960s for their efficacy against orthopoxviruses. They have had a lengthy history as prophylactic therapeutics with potential efficacy against *Mycobacterium tuberculosis*. However, it has become recently clear that this class of compounds has little, if any, potential for orthopoxvirus infections (i.e. cowpox virus, a surrogate virus for variola virus).¹²⁹

Several nucleoside analogues (i.e. S2242, 8-methyladenosine, idoxuridine) and nucleotide analogues (i.e. cidofovir, HPMPDAPy) have proven to be effective in various animal models of poxvirus infections.⁴⁸ In particular, cidofovir has shown high efficacy, even after administration of a single systemic (intraperitoneal) or intranasal (aerosolized) dose, in protecting mice from a lethal respiratory infection with either vaccinia or cowpox. Cidofovir has demonstrated high effectiveness in the treatment of disseminated progressive vaccinia in athymic-nude mice.¹¹⁵

In humans, cidofovir has been used successfully, by both the topical and intravenous route, in the treatment of orf and recalcitrant molluscum contagiosum in immunocompromised patients.³⁸ Cidofovir (HPMPC) and its congeners (HPMPDAP and HPMPDAPy) are highly effective against orf in human and ovine cell monolayers and organotypic ovine raft cultures.³¹ Given the *in vitro* activity of cidofovir against variola (smallpox) and other poxviruses, and the *in vivo* efficacy of cidofovir against various poxvirus infections in animal models and humans, it can be reasonably assumed that cidofovir should be effective in the therapy and/or prophylaxis of smallpox in case of an inadvertent outbreak or biological attack with the variola virus.

Being a phosphonate analogue, cidofovir only has limited oral bioavailability. In case of an outbreak of smallpox, it would be useful to have an orally active drug at hand.¹²² To this end, hexadecyloxypropyl-cidofovir (HDP-CDV) and octadecyloxyethyl-cidofovir (ODE-CDV) were

designed as potential oral prodrugs of cidofovir. These alkyloxyalkyl esters of cidofovir were found to significantly enhance inhibition of the replication of orthopoxviruses (i.e. vaccinia, cowpox) *in vitro*.⁸³ HDP-CDV and ODE-CDV given orally were as effective as cidofovir given parenterally for the treatment of vaccinia and cowpox infections.¹²⁸

HDP-CDV has proven effective in the treatment of a lethal vaccinia virus respiratory infection in mice.¹⁴⁰ Furthermore, HDP-CDV and ODE-CDV, when given orally, proved highly efficacious in a lethal (aerosol) mousepox (ectromelia) virus model,¹⁹ further attesting as to the potential usefulness of the alkyloxyalkyl esters of cidofovir in the oral therapy and prophylaxis of poxvirus infections. This potential usefulness has been recently extended to the alkoxyalkyl esters of (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) for the treatment of orthopoxvirus (i.e. vaccinia, cowpox) as well as cytomegalovirus infections.^{95,11}

In fact, as indicated by the most recent findings with cidofovir in mice infected with ectromelia (mousepox) virus encoding interleukin-4,¹³² and monkeys infected with monkeypox,¹⁴⁵ cidofovir (CDV) (and HDP-CDV and/or ODE-CDV) still provide the best current hope for effective control of virulent poxvirus infections.

Mutations in the E9L polymerase gene, i.e. A314T and A684V, of vaccinia virus have been associated with cidofovir resistance.³ Cidofovir resistance was associated with diminished virulence and reduced fitness *in vivo*, in mice.³ Cidofovir (CDV) still protected mice against CDV-resistant vaccinia virus.^{3,88}

In addition to the nucleotide analogue cidofovir, which primarily acts as a viral DNA chain terminator (for vaccinia virus DNA polymerase after it has been incorporated at the penultimate position),¹⁰⁵ antiviral strategies for poxvirus infections may also be based on inhibitors of cellular processes, i.e. signal transduction pathways. In this respect, the 4-anilinoquinazoline CI-1033, an ErbB tyrosine kinase inhibitor, was found to block variola virus replication *in vitro* and vaccinia virus infection *in vivo*.¹⁵⁹

Likewise, Gleevec® (STI-571, Imatinib), an Abl-family kinase inhibitor used to treat chronic myelogenous leukemia in humans was shown to suppress poxviral dissemination *in vivo* by several orders of magnitude and to promote survival in infected mice,¹³¹ suggesting possible use for this drug in treating smallpox or complications associated with vaccination against smallpox. Because the drug targets host rather than viral molecules, it is less likely to engender resistance compared to more specific antiviral agents. Collectively,^{159,131} inhibitors of

host-signaling pathways exploited by poxviral pathogens may represent potential antiviral therapies.

Recently, a new anti-poxvirus compound (ST-246) has been described, which is orally bioavailable, acts according to a novel mechanism of action, targeting a specific viral product (i.e. vaccinia virus F13L) required for extracellular virus particle formation and protecting mice from a lethal orthopoxvirus challenge.¹⁵⁸ These properties make ST-246 an attractive candidate for development as a smallpox antiviral drug that could be stockpiled for use in the treatment and prevention of smallpox virus infection in the event of a bioterrorist threat.

As already mentioned above, a wealth of nucleoside analogues, i.e. 5-substituted 2'-deoxyuridines (5-X-dUrds, related to idoxuridine) and neplanocin analogues, have been described as potent inhibitors of vaccinia virus [as the paradigm of poxviruses³⁷]. Several new congeners have been recently added to this list: i.e. 5-(dimethoxymethyl)-2'-deoxyuridine,⁵⁸ pyrazolone-pyrimidine-2'-deoxynucleoside chimera,⁵⁹ and a cyclopentenyl 1,2,3-triazole-4-carboxamide.²⁴ As has been demonstrated for many other viruses, small-interfering (si)RNAs have also proved effective against vaccinia virus, i.e. E3L-specific siRNAs targeting the double-stranded (ds)RNA binding protein E3L.³⁴

X. HEPADNAVIRUSES (HBV)

An estimated 400 million people worldwide are chronically infected with the hepadnavirus HBV; approximately 1 million die each year from complications of infection, including cirrhosis, hepatocellular carcinoma, and end-stage liver disease. Formally approved for the treatment of chronic hepatitis B are lamivudine, adefovir dipivoxil, (pegylated) interferon- α 2 and entecavir. Whereas lamivudine, adefovir and entecavir [and other nucleoside analogues which are still in (pre)clinical development] act as genuine antiviral agents at the HBV-associated reverse transcriptase, interferon, in the chronic hepatitis B setting, primarily acts as an immunomodulator.

Pegylated interferon- α 2b is effective in the treatment of HBeAg-positive chronic hepatitis B, but no additional benefit is achieved if it is combined with lamivudine.⁸¹ Nor does the addition of ribavirin seem to increase the efficacy of interferon in the treatment of HBeAg-positive chronic hepatitis B.¹⁰¹ Combination of pegylated interferon α -2b with adefovir dipivoxil, however, led to a marked decrease in serum HBV DNA and intrahepatic covalently closed circular DNA (cccDNA); which

was significantly correlated with HBsAg reduction in patients with chronic hepatitis B.¹⁵⁷

Whereas interferon therapy, also because of its unavoidable side effects (influenza-like symptoms) is not recommended for a duration longer than 1 year, the nucleos(t)ide analogues can, in principle, be administered for quite a number of years. For lamivudine (3TC), however, this prolonged treatment is compounded by the emergence of both virological and clinical resistance at an accumulating rate of approximately 20 percent of the patients per year (70% after 4 years of treatment). This resistance is primarily due to the emergence of the rt M204 I/V mutation in the YMDD motif of the HBV DNA polymerase [although, as has recently been demonstrated, lamivudine-resistant mutations can also emerge outside the YMDD motif].¹⁶¹

Resistance to adefovir dipivoxil may also emerge, but less frequently: not more than 6 percent after 3 years,⁷¹ although it may rise to 18 percent after 4 years¹⁰⁶ and 29% of patients after 5 years of therapy [as cited by Osborn and Lok].¹²⁰ Adefovir dipivoxil is the oral prodrug of adefovir [PMEA, 9-(2-phosphonylmethoxyethyl)adenine], which, after intracellular conversion to the diphosphate form, acts as a competitive inhibitor or alternative substrate for the HBV reverse transcriptase, and, when incorporated into the DNA, acts as a chain terminator, thereby preventing DNA chain elongation.⁴²

In patients with chronic HBV infection who were either positive¹⁰⁷ or negative⁷⁰ for hepatitis B e-antigen, 48 weeks of treatment with a dose of adefovir dipivoxil as low as 10 mg per day resulted in significant improvement of all parameters of the disease (histological liver abnormalities, serum HBV DNA titers and serum alanine aminotransferase levels). In patients with HBeAg-negative chronic hepatitis B, the benefits achieved from 48 weeks of adefovir dipivoxil were lost when treatment was discontinued, but maintained if treatment was continued through week 144.⁷¹ Adefovir dipivoxil (10 mg daily) treatment over 52 weeks proved safe and effective in Chinese subjects with HBeAg-positive chronic hepatitis B and during this period did not lead to emergence of drug resistance.¹⁶⁵

Resistance to adefovir dipivoxil is associated with the rt N236T and rt A181V/T mutations,¹²¹ as demonstrated in samples from patients with chronic HBV infection. Emergence of the rt A181V/T and rt N236T mutations is more common in lamivudine-resistant patients than in treatment-naïve patients.⁹⁸ Adefovir resistance can be associated with viral rebound, hepatitis flares and hepatic decompensation.⁶⁴ It has been suggested to combine lamivudine with adefovir dipivoxil, even in patients with lamivudine-resistant HBV, so as to

prevent emergence of adefovir resistance⁶⁵ In fact, adefovir dipivoxil should be added, i.e. in HBeAg-negative patients, to lamivudine as soon as genotypic resistance to lamivudine has developed.⁹⁴

Entecavir, one of the most recent antiviral drugs launched for clinical use, has *in vitro* and *in vivo* potency that seems to be greater than that of lamivudine: in patients with chronic hepatitis B infection it has proven efficacious at a dose as low as 0.5 mg per day.⁹¹ The active (triphosphate) metabolite of entecavir would accumulate intracellularly at concentrations that are inhibitory to 3TC-resistant HBV DNA polymerase.⁹⁹ It is not clear, however, how this translates to clinical efficacy of entecavir against lamivudine-resistant HBV infections. Early studies of entecavir indicate a low resistance potential, but resistance development over time must await the results of ongoing clinical trials.¹⁰⁸

Comprehensive studies with entecavir carried out in patients with either HBeAg-positive chronic hepatitis B²² or HBeAg-negative chronic hepatitis B⁹² pointed out that the rates of histologic improvement, virologic response, and normalization of alanine aminotransferase levels were significantly higher at 48 weeks of treatment with entecavir than with lamivudine.^{22,92} No case of resistance was detected after two years of entecavir therapy in patients who had not been previously treated with lamivudine. However, 10% of those patients that had failed on lamivudine therapy developed entecavir resistance after two years of therapy.¹⁷⁰

A number of L-nucleosides, i.e. β -L-thymidine (L-dT, Telbivudine), the 3'-valine ester of β -L-2'-deoxycytidine (Val-L-dC, Valtorcitabine) and 1-(2-fluoro-5-methyl- β -L-arabinosyl)uracil (L-FMAU, Clevudine) are in clinical development for the treatment of chronic hepatitis B [see Hu et al.⁷⁸]. As far as the role of deoxythymidylate (dTMP) kinase in the metabolism of clevudine is concerned, clevudine showed potent antiviral activity, which was sustained for 6 months after a 12-week treatment period in HBeAg-positive chronic hepatitis B patients.⁹⁶ Telbivudine did not show drug interaction with lamivudine or adefovir dipivoxil, which would allow combination of telbivudine with these drugs from a pharmacokinetics viewpoint.¹⁶⁸

Other compounds in preclinical development include 2',3'-dideoxy-3'-fluoroguanosine (FLG),⁸⁰ racivir and L-Fd4C. These compounds are also active against HIV (see Part II). FLG proved equally effective against wild-type, lamivudine-resistant and/or adefovir-resistant HBV mutants.⁸⁰ A new class of chemicals, represented by helioxanthin, has been recently described:¹⁶² these compounds would inhibit HBV repli-

cation by a mechanism of action that is different from any other anti-HBV agents described so far.²³

Moreover, tenofovir disoproxil fumarate (TDF) and emtricitabine [(–)FTC, the 5-fluoro-substituted counterpart of lamivudine)], which have both been licensed, individually and in combination, for the treatment of HIV infections (AIDS), may also be considered and further pursued, individually or in combination, for use in the treatment of chronic hepatitis B. TDF has been considered an important new therapeutic tool for the induction of complete remission in patients with lamivudine-resistant HBV infection;¹⁵⁰ it may be a highly effective rescue drug for HBV-infected patients with diminished responsiveness to treatment with lamivudine and adefovir dipivoxil.¹⁵¹ At the dose used for the treatment of HIV infections, that is 300 mg/day, TDF has been found effective against wild-type and lamivudine-resistant HBV strains in HBV/HIV-coinfected patients.¹²

Like TDF, emtricitabine [(–)FTC] has activity against both HIV and HBV, and should, therefore, be considered for use in patients coinfected with HIV and HBV.¹³⁵ An interesting recommendation has been proposed for the care of patients with chronic HBV and HIV co-infection.¹⁴³ They should be put on the combination of TDF with (–)FTC, which would cover both the HBV and HIV infection. Only if no antiretroviral therapy would be used in these patients, adefovir dipivoxil and/or pegylated interferon may be installed depending on whether they are HBeAg-negative or -positive, respectively.¹⁴³

As has been shown for many other viruses, the RNA interference (RNAi) approach based on short interfering RNAs (siRNAs) can also be applied to specifically inhibit HBV replication *in vitro*, in cell culture,^{163,124} and *in vivo*, in mice transfected with an HBV plasmid.¹⁰⁹ In fact, several studies have demonstrated that siRNAs are capable of specifically inhibiting HBV replication *in vivo*^{109,86,67} and thus may constitute a new therapeutic strategy for HBV infection.

XI. RETROVIRUSES (HIV)

There are at present twenty some compounds available for the treatment of HIV infections.⁴⁵ These compounds fall into 5 categories: (i) nucleoside reverse transcriptase inhibitors (NRTIs): zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and emtricitabine; (ii) nucleotide reverse transcriptase inhibitors (NtRTIs): tenofovir disoproxil fumarate; (iii) non-nucleoside reverse transcriptase inhibitors (NNRTIs): nevirapine, delavirdine and efavirenz; (iv) protease

inhibitors (PIs): saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir (combined at a 4 to 1 ratio with ritonavir), atazanavir, fosamprenavir, tipranavir and darunavir; and (v) fusion inhibitors (FIs): enfuvirtide. Several of these compounds are also available as fixed dose combinations: zidovudine with lamivudine (Combivir®), lamivudine with abacavir (Kivexa®), and emtricitabine with tenofovir disoproxil fumarate (Truvada®). A triple-drug fixed dose combination, containing efavirenz, emtricitabine and tenofovir disoproxil fumarate (Atripla®) has recently been launched.

In addition to the 22 licensed anti-HIV compounds, various others are (or have been) in clinical [phase II (or III)] development: the HIV-1 attachment inhibitors BMS-378806 and BMS-488043,¹⁰⁴ the CXCR4 antagonist AMD-3100 (as stem cell mobilizer for stem cell transplantation in patients with non-Hodgkin lymphoma or multiple myeloma),⁴³ the CCR5 antagonists¹⁵⁴ SCH-C, vicriviroc (SCH-D, SCH 417690),¹⁴⁶ aplaviroc (873140)¹⁵³ and maraviroc (UK-427,857),⁵⁶ the NRTIs racivir, (–)-DOTC [AVX-754 (SPD-754), which has been accredited with activity against most other NRTI-resistant HIV-1 strains],⁶⁹ reverset, elvucitabine, alovudine and amdoxovir, the NNR-TIs capravirine and etravirine, the protease inhibitor (PI) darunavir (TMC-114)^{45,52} (which, in the mean time, has been approved for clinical use), and the gag (p24) maturation inhibitor 3-O-(3',3'-dimethylsuccinyl)-betulinic acid (PA-457).¹⁰⁰ Also, a prodrug of the benzophenone GW 678248 has recently progressed to phase II clinical studies.¹³³

The protease inhibitor brecanavir (GW 640385) has progressed to phase II/III clinical trials.¹⁴⁴ Although brecanavir can engender, on its own, *in vitro* drug resistance development,¹⁶⁰ co-administration of brecanavir (300 mg) with ritonavir (100 mg) significantly increased the plasma brecanavir levels, achieving drug concentration predicted to inhibit protease inhibitor-resistant HIV mutants.⁶³ Pharmacokinetic boosting with sub-therapeutic doses of ritonavir has become a standard procedure to enhance systemic drug exposures for a variety of the HIV protease inhibitors.

Yet other compounds are in preclinical development and/or may soon proceed to clinical phase I/II clinical studies: the CD4 (HIV receptor) down-modulator cyclotriazadisulfonamide (CADA),¹⁵² the HIV gp120 envelope-binding protein cyanovirin-N as a topical microbicide;¹⁸ KRH-2731, a CXCR4 antagonist, structurally related to KRH-1636;⁷⁹ the CXCR4 antagonist AMD-070 (a derivative of the bicyclam AMD3100, which is currently being pursued in phase II/III clinical trials, in combination with granulocyte colony-stimulating factor (G-CSF), for the mobilization of autologous hematopoietic progen-

Table 1. The past, present and future of antiviral drugs (Part I: DNA viruses and retroviruses)

Virus	Compound		
	Approved for medical use	In clinical development	In preclinical evaluation
Parvo (B19)	–	–	–
Polyoma (JC, BK)	Cidofovir (“off label”)	–	–
Papillomas (HPV)	Cidofovir (“off label”)	–	cPr PMEDAP and other acyclic nucleoside phosphonates Biphenylsulfonacetic acid derivatives
Adeno	Cidofovir (“off label”)	–	HPMPO-DAPy HDP-HPMPA, ODE-HPMPA
α -Herpes (HSV-1, HSV-2, VZV)	Acyclovir Valaciclovir Penciclovir (topical) Famciclovir Brivudin Idoxuridine (topical) Trifluridine (topical)	H2G prodrug	Tricyclic acyclovir derivatives A-5021 Synguanol Cyclopropavir (ZSM-I-62) BILS 45 BS BAY 57-1293 BCNA Cf 1742 BCNA Cf 1743
β -Herpes (CMV, HHV-6, HHV-7)	Ganciclovir Valganciclovir Cidofovir Foscarnet Fomivirsen	Maribavir	CMV423 HDP-CDV (CMX001) ODE-CDV 3-Deaza-HPMPA
γ -Herpes (EBV, HHV-8)	Cidofovir (“off label”)	–	<i>North</i> -methanocarbathymidine (N-MCT)

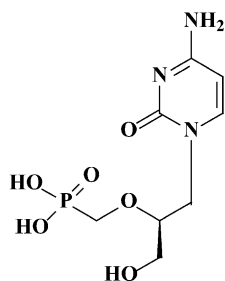
Table 1. — *Continued*

Virus	Compound		
	Approved for medical use	In clinical development	In preclinical evaluation
Pox (Variola, Vaccinia, Monkeypox, Molluscum contagiosum, orf, ...)	Cidofovir ("off label)	—	HPMPO-DAPy HDP-CDV, ODE-CDV HDP-HPMPA, ODE-HPMPA CI-1033 ST-246 5-X-dUrds Pyrazolone-pyrimidine 2'-deoxy-nucleoside chimera Cyclopentenyl 1,2,3-triazole-4-carboxamide 3'-Fluoro-2',3'-dideoxyguanosine Helioxanthin
Hepadna (HBV)	Lamivudine Adefovir dipivoxil Entecavir Pegylated interferon- α Telbivudine	Valtorcitabine Clevudine	
Retro (HIV)	Zidovudine Didanosine Zalcitabine Stavudine Lamivudine Abacavir Emtricitabine	BMS-378806 BMS-488043 AMD-3100 SCH-C Vicriviroc Aplaviroc Maraviroc	Cyclotriazadisulfonamide Cyanovirin N KRH-1636 TAK-779 TAK-220 TAK-652 MIV-210

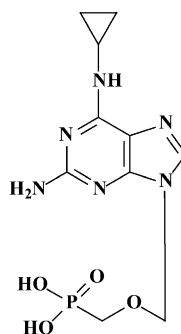
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Table 1. — Continued

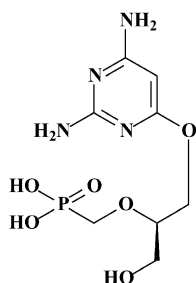
Virus	Compound		
	Approved for medical use	In clinical development	In preclinical evaluation
	Tenofovir disoproxil fumarate	Racivir	DOT
	Nevirapine	AVX-754	4'-Ed4T
	Delavirdine	Reverset	PMEO-DAPy
	Efavirenz	Elvucitabine	PMPO-DAPy
	Saquinavir	Alovudine	PMDTA
	Ritonavir	Amdoxovir	PMDTT
	Indinavir	Capravirine	HDP-HPMPA, ODE-HPMPA
	Nelfinavir	Etravirine	Thiocarboxanilide UC-781
	Amprenavir	Brecanavir	Dapivirine
	Lopinavir	PA-457	Rilpivirine
	Atazanavir	GW678248	L-870810
	Fosamprenavir		L-870812
	Tipranavir		GS-9137 (JTK-303)
	Darunavir		Dihydroxytropolone
	Enfuvirtide		Pyrimidinyl diketo acid
			Indolyl aryl sulfone
			Pradimicin A



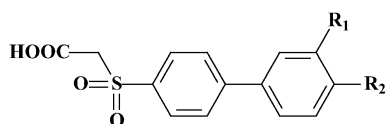
HPMPC
Cidofovir
Vistide®



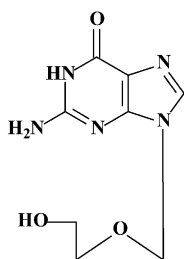
cPrPMEDAP



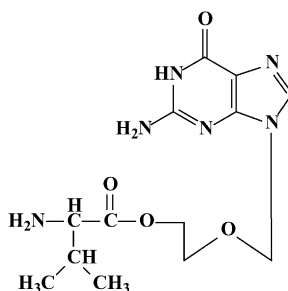
HPMPO-DAPy



Biphenylsulfonacetic acid

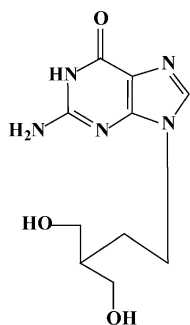


Acyclovir (ACV)
Zovirax®

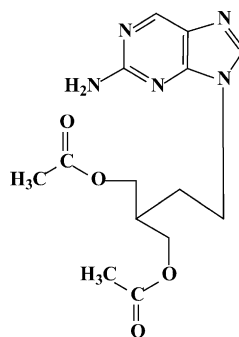


Valaciclovir (VACV)
Zelitrex®, Valtrex®

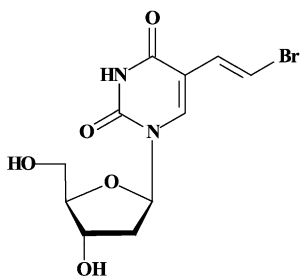
Figure 1. Structural formulae of antiviral compounds.



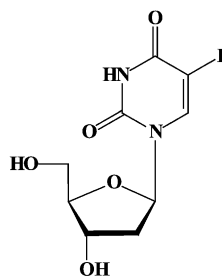
Penciclovir (PCV)
Denavir®, Vectavir®



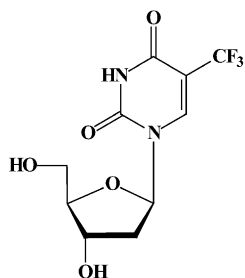
Famciclovir (FCV)
Famvir®



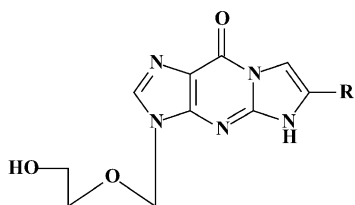
(E)-5-(2-Bromovinyl)-2'-deoxyuridine
BVDU
Brivudin
Zostex®, Brivirac®, Zerpex®



Idoxuridine
Herpid®, Stoxil®

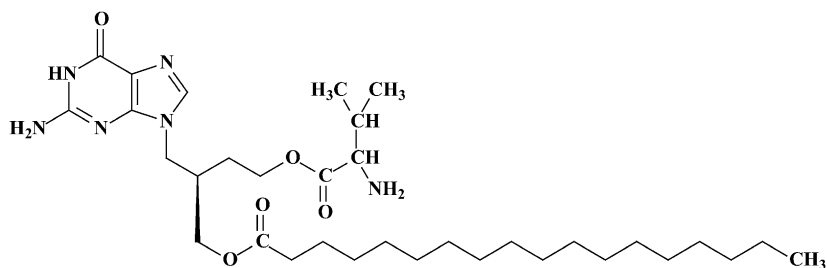


Trifluridine
Viroptic®

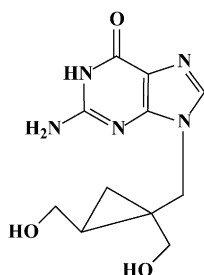


Tricyclic acyclovir derivatives

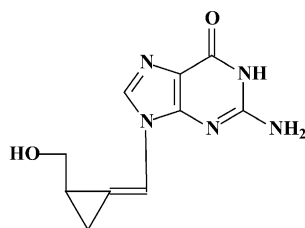
Figure 1. — *Continued.*



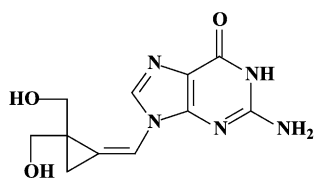
H2G prodrug
MIV-606
Valomaciclovir stearate



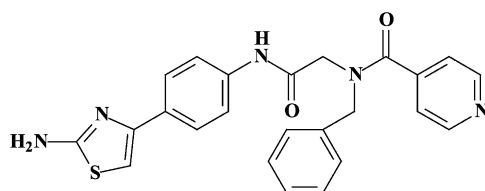
A-5021



Synguanol



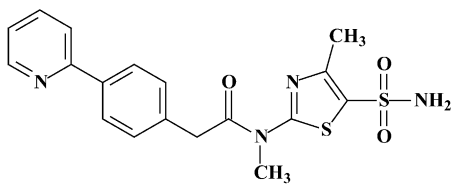
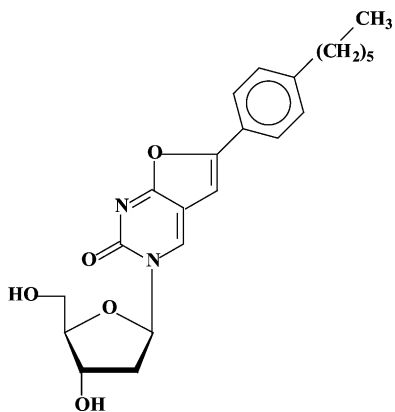
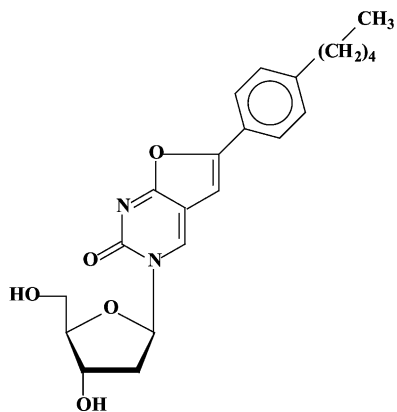
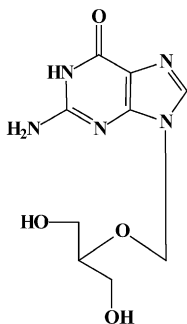
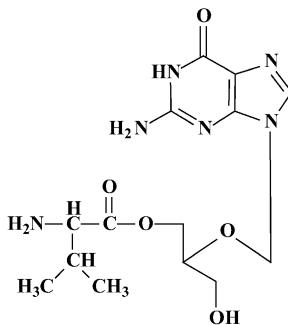
Cyclopropavir
(ZSM-I-62)

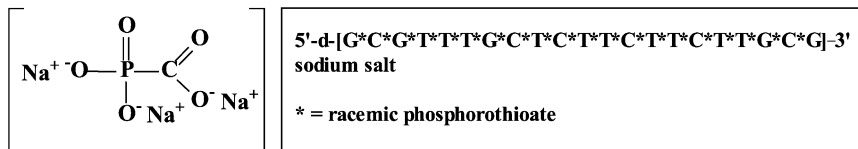


BILS 45 BS

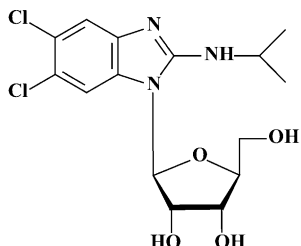
Figure 1. — *Continued.*

itor cells),⁶² TAK-220, a CCR5 antagonist, structurally related to TAK-779,⁵ which has proved to be a highly potent (orally bioavailable) inhibitor of CCR5-using (R5) HIV-1 strains,^{147,116} and acts synergisti-

**BAY 57-1293****BCNA Cf 1742****BCNA Cf 1743****Ganciclovir**
Cymevene®, Cytovene®**Valganciclovir**
Valcyte®*Figure 1. — Continued.*

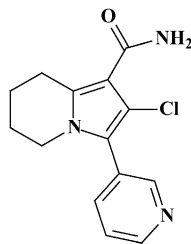


Foscarnet
Foscavir®

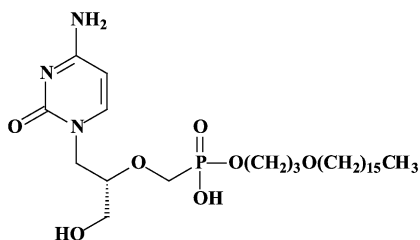


1263W94
Maribavir

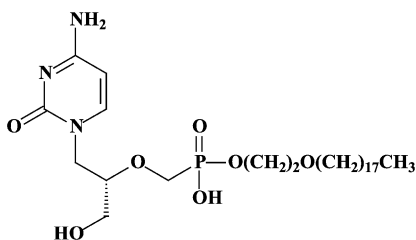
Fomivirsen
Vitravene®



CMV423



HDP-CDV
(CMX001)

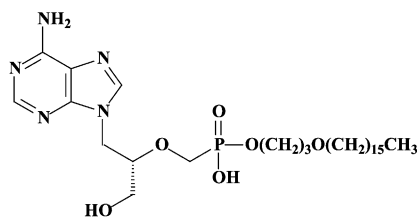
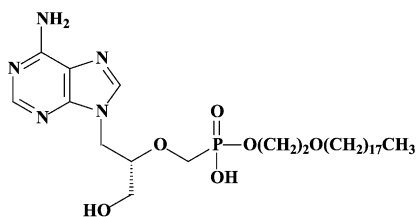
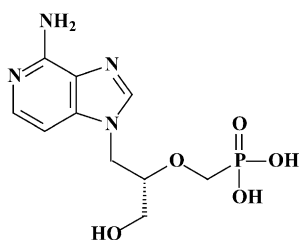
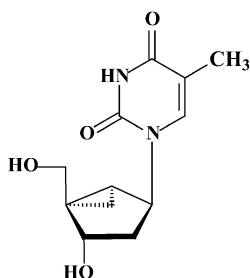
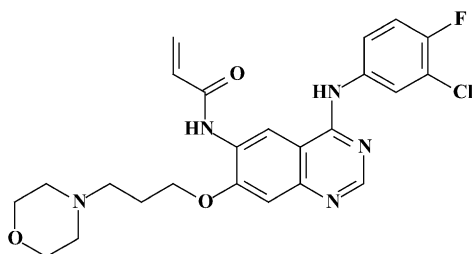


ODE-CDV

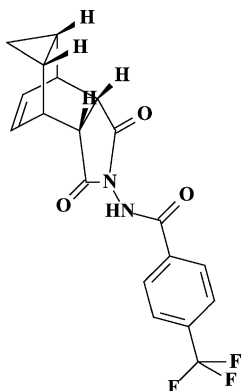
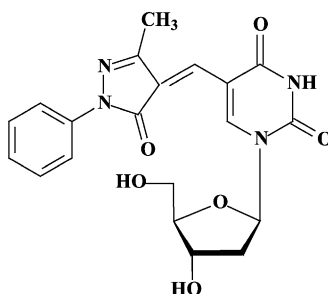
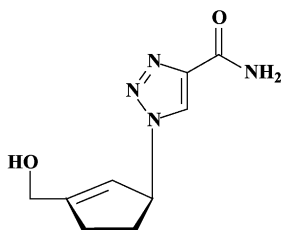
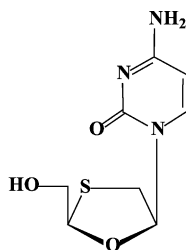
Figure 1. — Continued.

cally with other antiretrovirals;¹⁴⁹ TAK-652, another orally bioavailable inhibitor of CCR5-mediated HIV infection.⁶

Noteworthy among the new NRTIs are MIV-210, a prodrug of the NRTI 3'-fluoro-2',3'-dideoxyguanosine; the thymine dioxolane DOT, another NRTI;²⁷ 4'-Ed4T (2',3'-didehydro-3'-deoxy-4'-ethynyl-2'-deoxythymidine), which has favorable oral bioavailability and a unique drug resistance profile, different from that of the other NRTIs.¹¹⁷ Newly synthesized "phosphonate" analogues include the NtRTIs

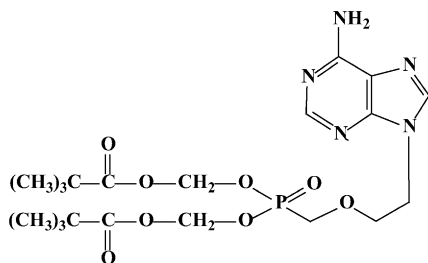
**HDP-HPMPA****ODE-HPMPA****3-Deaza-HPMPA****(N)-MCT**
North-Methanocarbathymidine**Anilinoquinazoline**
CI-1033**Figure 1.** — *Continued.*

6-[2-(phosphonomethoxy)alkoxy]-2,4-diaminopyrimidines PMPO-DAPy, PMEO-DAPy, and 5-substituted derivatives thereof,⁵¹ and the deoxythreosyl nucleoside phosphonates phosphonomethyldeoxythreosyladenine (PMDTA) and -thymine (PMDTT).¹⁵⁶ Also alkoxyalkyl [i.e. hexadecyloxypropyl (HDP) and octadecyloxyethyl (ODE)] esters of the prototype “phosphonate”, (S)-9-[3-hydroxy-2-(phosphonomethoxy)-

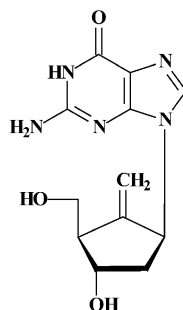
**ST-246****Pyrazolone-pyrimidine
2'-deoxynucleoside chimera****Cyclopentenyl 1,2,3-triazole-4-
carboxamide****Lamivudine
(-)-β-L-3'-Thia-2',3'-dideoxycytidine
(3TC)
Epivir®, Zeffix®****Figure 1. — Continued.**

propyl]-adenine [HPMPA] have been reported to inhibit HIV-1 replication *in vitro* at nanomolar concentrations.⁷⁷

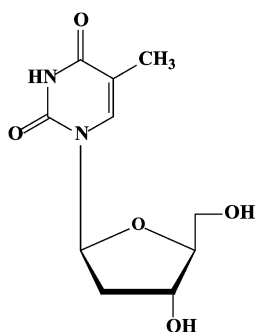
Among the NNRTIs which have been further pursued for their anti-HIV potential, are thiocarboxanilide UC-781 and dapivirine (TMC-120), both as topical microbicides, and rilpivirine (R-278474), one of the most potent anti-HIV agents ever described;⁸² GW678248, a novel benzophenone NNRTI,^{61,74} which has activity at 1 nM against the K103N and Y181C RT HIV-1 mutants associated with clinical resistance to efavirenz and nevirapine, respectively,¹³³ the alkenyldiarylmethanes



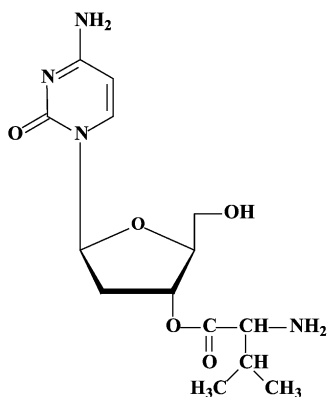
Adefovir dipivoxil
Bis(pivaloyloxymethyl)ester of
9-(2-phosphonylmethoxyethyl)adenine,
bis(POM)PMEA
Hepsera®



Entecavir
Baraclude®



L-dT
Telbivudine
Tyzeka®, Sebivo®

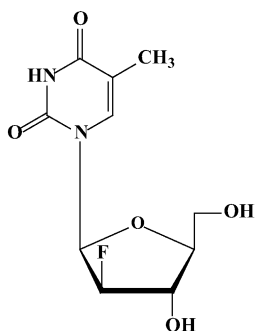


3'-Val-L-dC
3'-Valine ester of β-L-deoxycytidine
Valtorcitabine

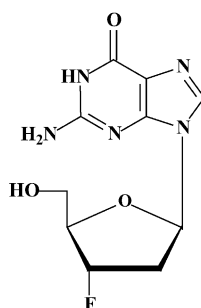
Figure 1. — Continued.

(ADAMS) with metabolically labile methylester moieties replaced by isoxazolone, isoxazole, oxazolone or cyano substituents.⁵⁴

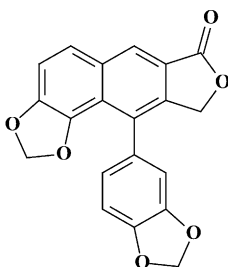
The polymerase activity of the HIV-1 reverse transcriptase (RT) is entirely dependent on the heterodimeric structure of the enzyme. RT dimerization, therefore, represents a molecular target for the development of new HIV inhibitors; it is the point of attack for the 2',5'-bis-*O*-



L-FMAU
Clevudine



FLG
3'-Fluoro-2',3'-dideoxyguanosine

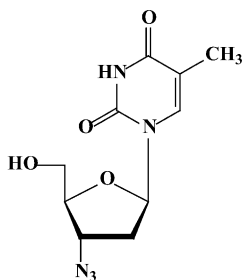


Helioxanthin

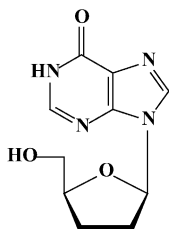
Figure 1. — *Continued.*

tert-butyldimethylsilyl- β -D-ribofuranosyl]-3'-spiro-5''-(4''-amino-1'',2''-oxathiole-2'',2''-dioxide)-thymine (TSAO-T) derivatives, a class of compounds originally categorized under the NNRTIs.¹³⁹

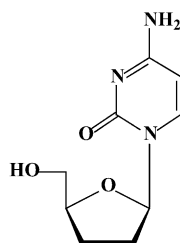
A number of compounds, among which the 1,6-naphthyridine-7-carboxamides L-870,810 and L-870,812, are targeted at the HIV-1 integrase.^{75,76} Novel HIV-1 integrase inhibitors have been derived from quinolone antibiotic.¹³⁶ Phase I/II clinical studies have been undertaken with GS-9137 (JTK-303), the prototype of this class of compounds.⁵³ This compound showed an EC_{50} of 0.9 nM in an acute HIV-1 infection assay,¹³⁶ and effected a $2\log_{10}$ reduction in viral load in short-term trials in short-term monotherapy trials.⁵³ The 3,7-dihydroxytropolones represent an interesting platform for the design of inhibitors of both the reverse transcriptase (and RNase H) as well



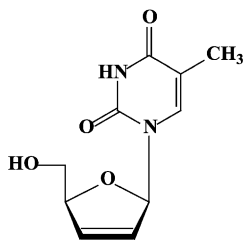
Zidovudine
3'-Azido-2',3'-dideoxythymidine, azidothymidine (AZT)
Retrovir®



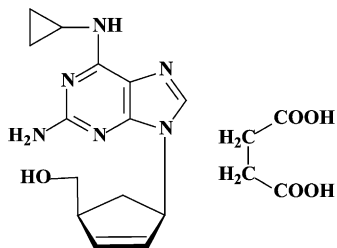
Didanosine
2',3'-Dideoxyinosine (ddI)
Videx®, Videx® EC



Zalcitabine
2',3'-Dideoxycytidine (ddC)
Hivid®

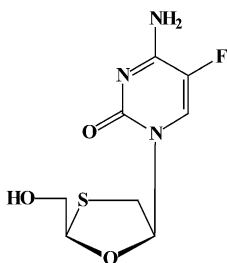


Stavudine
2',3'-Didehydro-2',3'-dideoxythymidine (d4T)
Zerit®

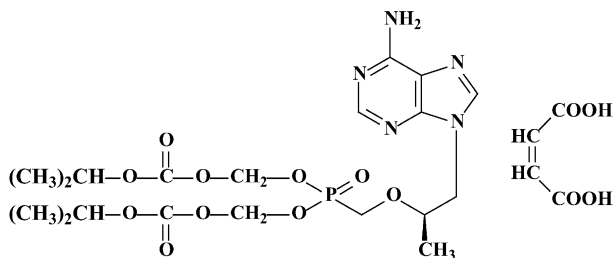


Abacavir
(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]-2-cyclopentene-1-methanol succinate (ABC)
Ziagen®

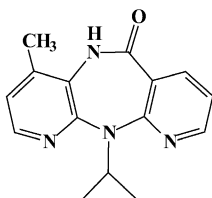
Figure 1. — *Continued.*



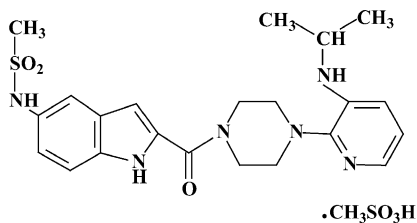
Emtricitabine
 (-)-β-L-3'-thia-2',3'-dideoxy-5-fluorocytidine [(-)-FTC]
 Emtriva®



Tenofovir disoproxil fumarate (TDF)
 Fumarate salt of bis(isopropoxycarbonyloxymethyl) ester of
 (*R*)-9-(2-phosphonylmethoxypropyl)adenine
 bis(POC)-PMPA
 Viread®

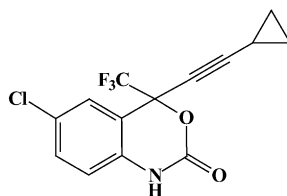


Nevirapine
 Viramune®

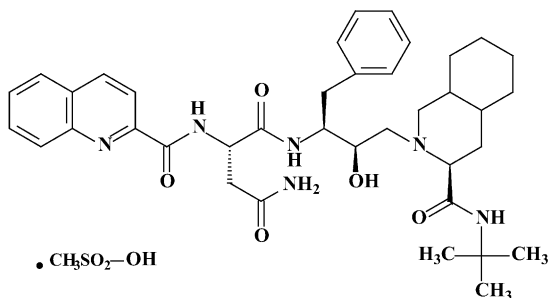


Delavirdine
 Rescriptor®

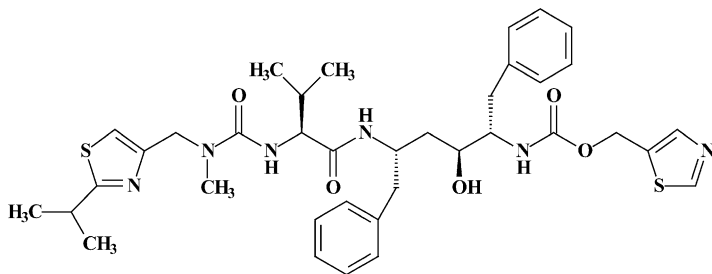
Figure 1. — *Continued.*



Efavirenz
Sustiva®, **Stocrin®**



Saquinavir
 hard gel capsules, **Invirase®**
 soft gelatin capsules, **Fortovase®**



Ritonavir
Norvir®

Figure 1. — Continued.

as the HIV integrase.⁵⁵ Similarly, indolyl aryl sulfone may serve as a platform for the design of new NNRTIs effective against K103N HIV-1 variants.²⁰ Recently, diketo acids bearing a nucleobase scaffold have

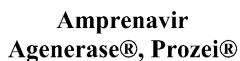
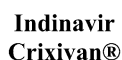
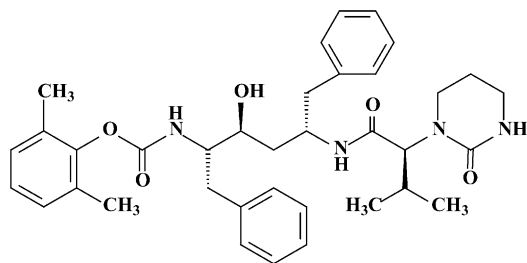
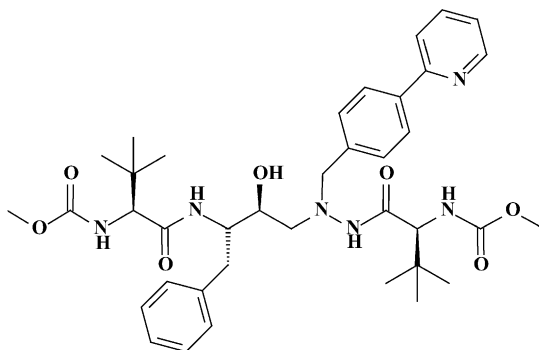


Figure 1. — *Continued.*

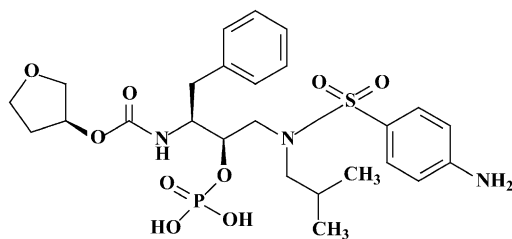
been described as highly potent HIV integrase inhibitors:¹¹⁴ the prototype compound, 4-(1,3-dibenzyl-1,2,3,4-tetrahydro-2,4-dioxypyrimidin-5-yl)-2-hydroxy-4-oxo-but-2-enoic acid, exhibited an anti-HIV selectiv-



Lopinavir
combined with ritonavir at 4/1 ratio
Kaletra®

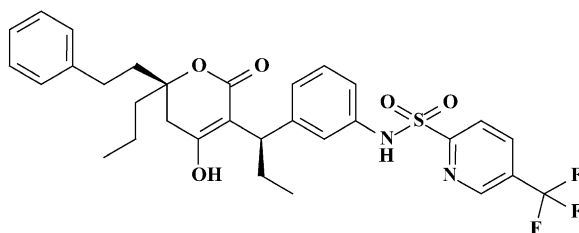


Atazanavir
Reyataz®

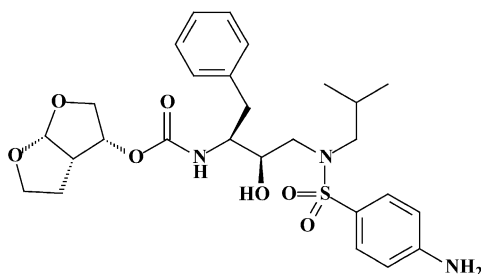


Fosamprenavir
Lexiva®, Telzir®

Figure 1. — Continued.



Tipranavir (U-140690)
Aptivus®



Darunavir (TMC-114)
Prezista®

Figure 1. — *Continued.*

ity index in cell culture of >4000. Meanwhile, an HIV vector-based single-cycle assay has been developed which should facilitate the evaluation of potential HIV integrase inhibitors.¹⁷

Among the PIs, novel HIV-1 protease inhibitors have been described which were designed specifically to interact with the backbone of HIV protease active site to combat drug resistance,⁶⁶ and, among the triterpene (betulinic acid) derivatives new potent anti-HIV agents were reported¹⁶⁴ to demonstrate a better antiviral profile than the prototype compound PA-457.¹⁰⁰

In addition to the aforementioned cyanovirin-N, thiocarboxanilide UC-781 and dapivirine, there are some other compounds that could be further developed as topical (i.e. vaginal) microbicides, namely the aglycons of the glycopeptide antibiotics vancomycin, teicoplanin and eremomycin which specifically interact with the gp120 glycoprotein.¹²⁶ Also the plant lectins, i.e. *Galanthus nivalis* agglutinin (GNA) and *Hippeastrum* hybrid agglutinin (HHA) represent potential candidate anti-

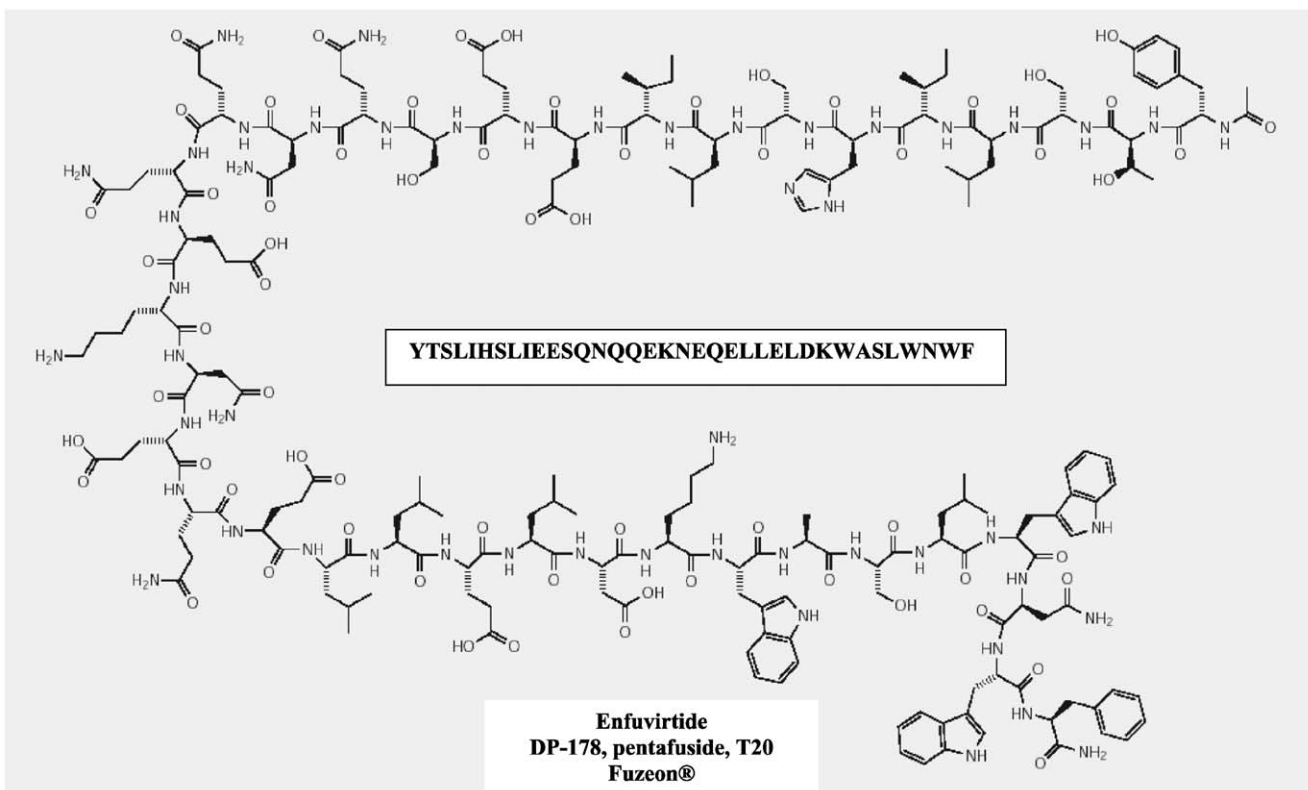
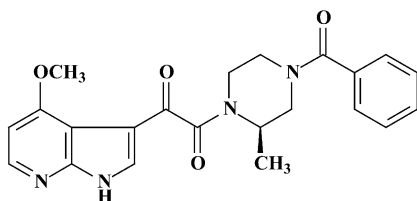
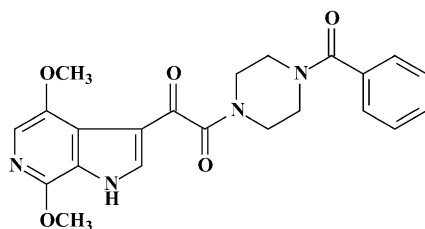
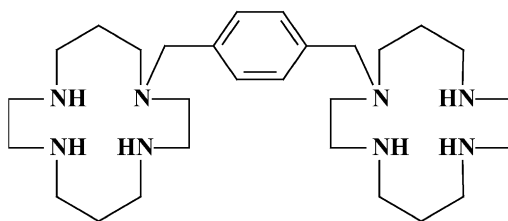
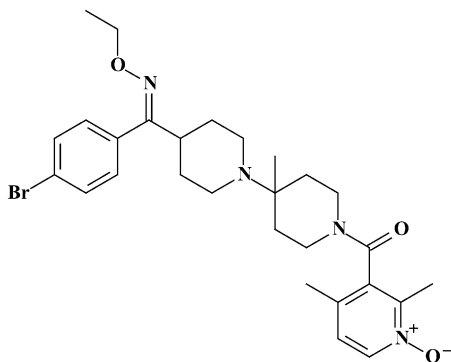
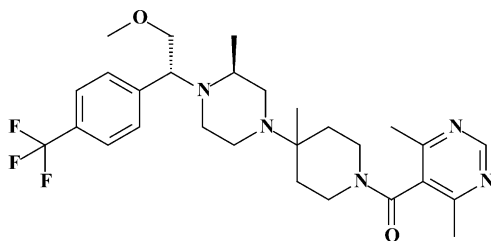
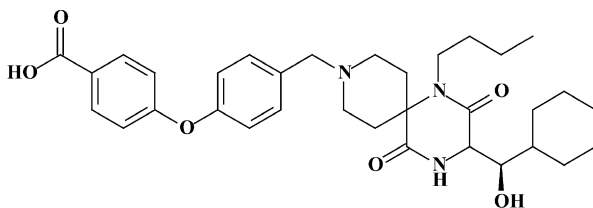


Figure 1. — Continued.

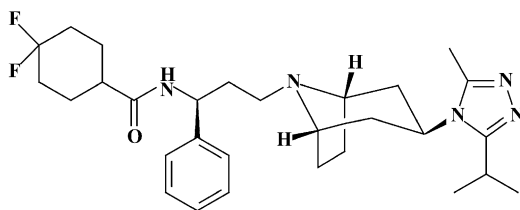
**BMS-378806****BMS-488043****AMD 3100**
Mozobil™**Figure 1.** — *Continued.*

HIV microbicides: they show marked stability at relatively low pH and high temperatures for prolonged time periods, they directly interact with the viral envelope and prevent entry of HIV into its target cells.⁷ Upon prolonged exposure of HIV in cell culture to HHA or GNA, the virus acquires resistance mutations in the gp120 glycoprotein which are predominantly located at the N-glycosylation (asparagine) sites.⁸

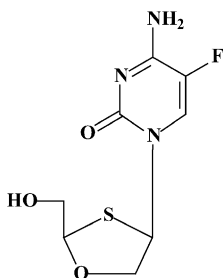
Cyanovirin-N and the plant lectins GNA and HHA can be termed carbohydrate-binding agents (CBAs); due to their carbohydrate-binding properties, they interact with the viral envelope glycoprotein, thereby preventing the HIV entry process. Recently, a non-peptidic benzon-

**SCH-C (SCH-351125)****SCH-D (SCH-417690)**
Vicriviroc**873140 (GW-873140, ONO-4128, AK-602)**
Aplaviroc**Figure 1.** — *Continued.*

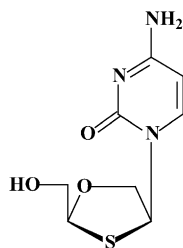
aphtacene quinone antibiotic, pradimicin A, has been described as a low-molecular-weight CBA that blocks HIV entry by specifically interacting with the mannose moieties of the HIV-1 gp120.⁹



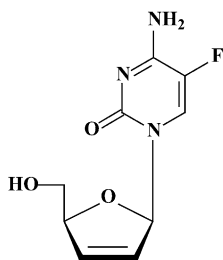
UK-427857
Maraviroc



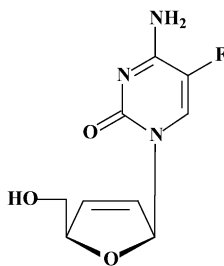
Racemic (±)FTC (FdOTC)
Racivir®



AVX-754 ((-)-dOTC)



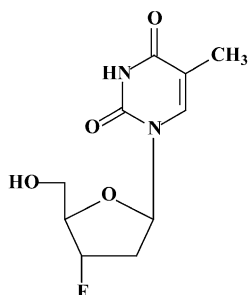
DPC-817 (β-D-Fd4C)
Reverset®



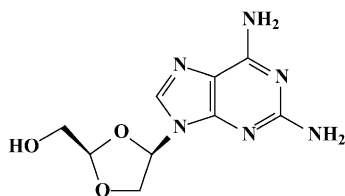
ACH-126443 (β-L-Fd4C)
Elvucitabine

Figure 1. — Continued.

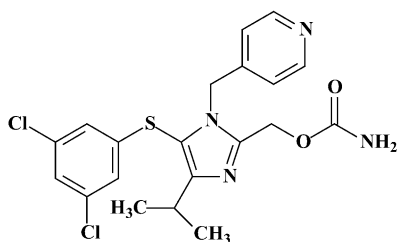
An avenue to be further explored is the combination of different microbicides, such as the NNRTI thiocarboxanilide UC-781 with the cellulose acetate 1,2-benzenedicarboxylate (CAP) viral entry inhibitor, which exhibit synergistic and complementary effects against HIV-1 in-



MIV-310 (FddThd, FLT)
Alovudine



Diaminopurine dioxolane (DAPD)
Amdoxovir

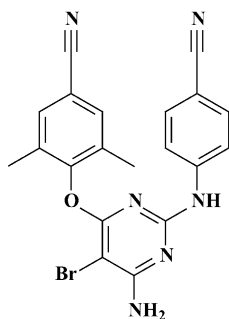
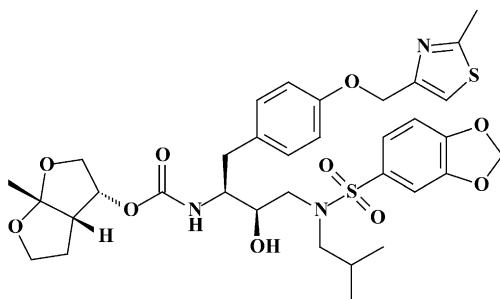
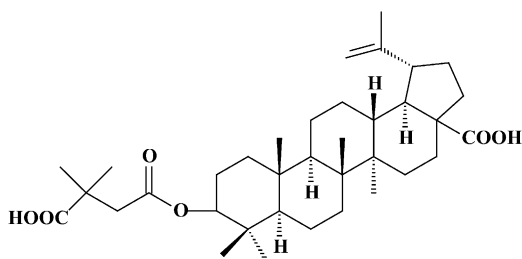


Capravirine (S-1153, AG1549)

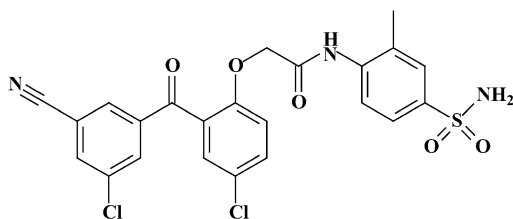
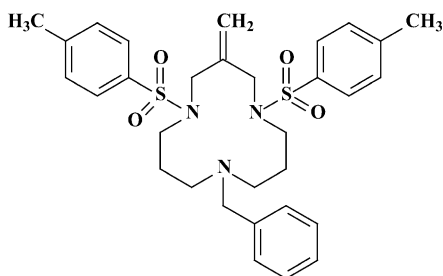
Figure 1. — *Continued.*

fection.¹⁰² There is, in addition, no shortage of sulfated and sulfonated polymers (starting off with suramin, the first polysulfonate ever shown to be active against HIV) which could be considered as topical anti-HIV microbicides.¹³⁷

RNA interference (RNAi) may be considered a new powerful tool for intracellular immunization against HIV-1 infection. It has been demonstrated in short-term assays that HIV-1 replication can be inhibited by siRNAs directed against viral targets (i.e. *rev*) or cellular targets (i.e. CCR5).^{4,97,119,127} As to the viral targets, siRNAs targeting conserved *gag*, *pol*, *int*, *vpv* regions²¹ or gp41, *tat*, *rev* or *nef*³³ were shown to inhibit virus production. Although targeting single HIV-1 sequences with siRNAs can result in strong inhibition of viral replication, it is likely followed by the escape of mutated viral variants.³² Therefore, antiviral approaches involving RNAi should be used in a combined fashion so as to prevent emergence of resistant viruses.

**Etravirine (TMC-125, R-165335)****Brecanavir (GW640385)****PA-457****Figure 1.** — *Continued.*

It is, furthermore, worth investigating whether RNAi can be harnessed for use in microbicides. As described above, an siRNA-based microbicide has been shown to protect mice from lethal HSV-2 in-

**GW678248****Cyclotriazadisulfonamide
CADA***Figure 1. — Continued.*

fection.¹²³ Extension of these results to the design of an HIV microbicide would also require demonstrating silencing in resident tissue macrophages, dendritic cells and T cells. Further considering the requirement of combining siRNAs that target multiple viral genes so as to cover viral sequence diversity and to prevent potential escape mutation, the development of an effective siRNA-based HIV microbicide may seem as a challenging task.

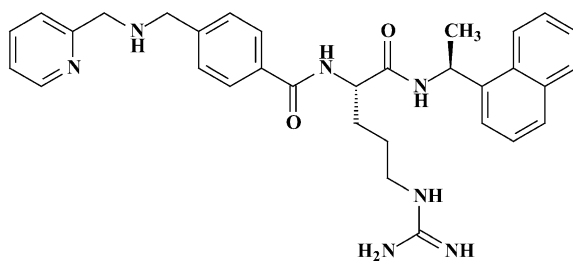
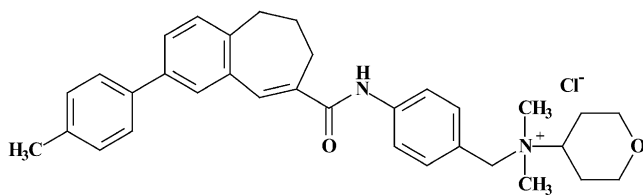
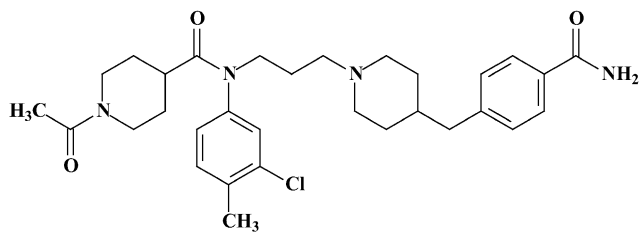
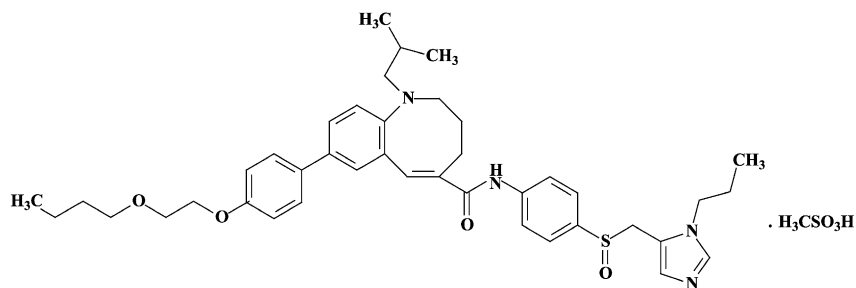
XII. CONCLUSION

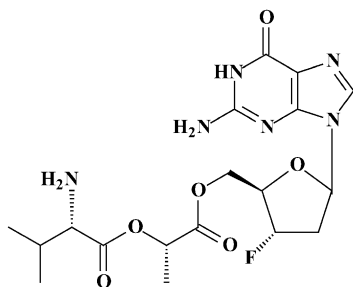
About forty compounds are registered as antiviral drugs, at least half of which are used to treat HIV infections. An even greater number of compounds are under clinical or preclinical development, with again, as many targeting HIV as all the other viruses taken together. This implies that HIV, since its advent, has remained the main stay

(H_2N) 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)

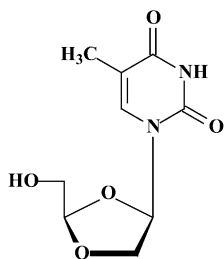
Cyanovirin-N

Figure 1. — Continued.

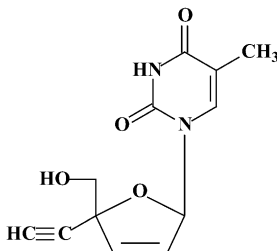
**KRH-1636****TAK-779****TAK-220****TAK-652****Figure 1.** — Continued.



MIV-210 [FLG (3'-fluoro-2',3'-dideoxyguanosine) prodrug]



**1-(β-D-dioxolane)thymine
DOT**



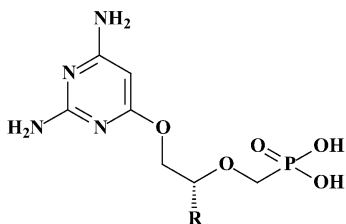
4'-Ed4T

Figure 1. — Continued.

in antiviral drug development. Antiviral agents can, as guided by the anti-HIV agents as examples, be divided in roughly five categories:

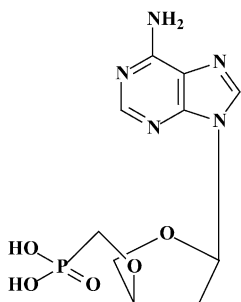
- (i) nucleoside analogues,
- (ii) nucleotide analogues (or acyclic nucleoside phosphonates),
- (iii) non-nucleoside analogues,
- (iv) protease inhibitors, and
- (v) virus–cell fusion inhibitors.

Molecular targets are for (i) and (ii) the viral DNA polymerase (whether DNA-dependent as in the case of herpesviruses, or RNA-dependent as in the case of HIV or HBV); for (iii) RNA-dependent DNA polymerase (reverse transcriptase), associated with HIV, or RNA-dependent RNA polymerase (RNA replicase) associated with HCV; for (iv) the proteases associated with HIV and HCV; and for (v) the fusion

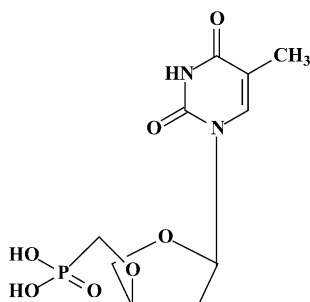


$R = \text{CH}_3$: PMPO-DAPy

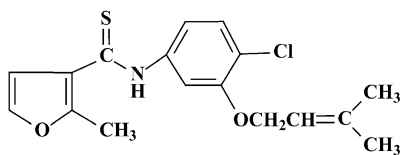
$R = \text{H}$: PMEO-DAPy



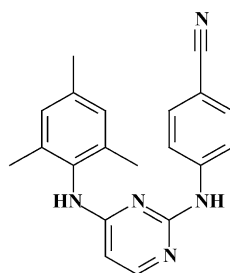
PMDTA



PMDTT



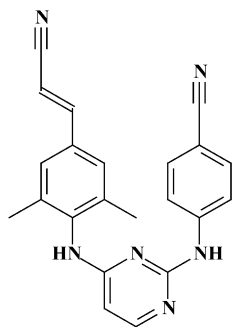
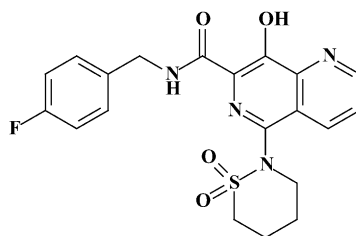
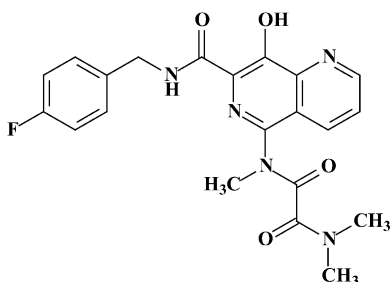
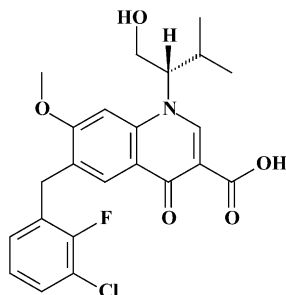
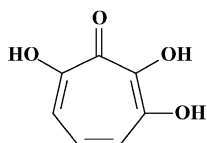
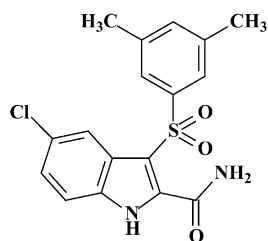
Thiocarboxanilide UC-781



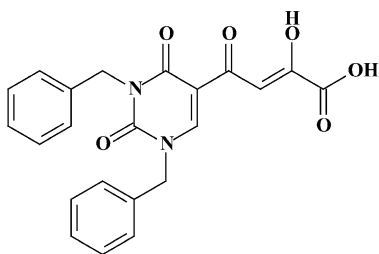
Dapivirine (TMC-120, R-147681)

Figure 1. — *Continued.*

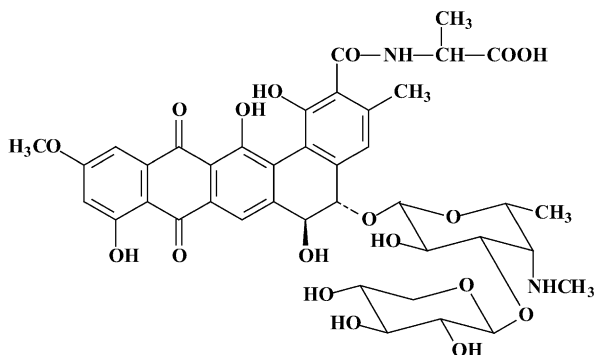
process of HIV (and, potentially, other viruses such as the SARS coronavirus and RSV). Antiviral agents may also exert their antiviral effects through an interaction with cellular targets such as IMP dehydrogenase (ribavirin) and SAH hydrolase (3-deazaneplanocin A). The latter

**Rilpivirine (R-278474)****L-870810****L-870812****GS-9137 (JTK-303)****3,7-dihydroxytropolone****Indolyl aryl sulfone
RS 1588***Figure 1. — Continued.*

enzymes are essential for viral RNA synthesis (through the supply of GTP) and viral mRNA maturation (through 5'-capping), respectively. Finally, interferons (now generally provided in their pegylated form)



**4-(1,3-dibenzyl-1,2,3,4-tetrahydro-2,4-dioxypyrimidin-5-yl)-
2-hydroxy-4-oxo-but-2-enoic acid**



Pradimicin A

Figure 1. — Continued.

may be advocated in the therapy of those viral infections (actually, HBV and HCV; prospectively, Coxsackie B, SARS, . . .) that, as yet, cannot be sufficiently curbed by other therapeutic measures.

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STATUS PRESENTS OF ANTIVIRAL DRUGS AND STRATEGIES: PART II: RNA VIRUSES (EXCEPT RETROVIRUSES)

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ABSTRACT

More than 40 compounds have been formally licensed for clinical use as antiviral drugs, and half of these are used for the treatment of HIV infections. The others have been approved for the therapy of herpesvirus (HSV, VZV, CMV), hepadnavirus (HBV), hepacivirus (HCV) and myxovirus (influenza, RSV) infections. New compounds are in clinical development or under preclinical evaluation, and, again, half of these are targeting HIV infections. Yet, quite a number of important viral pathogens (i.e. HPV, HCV, hemorrhagic fever viruses) remain in need of effective and/or improved antiviral therapies.

I. INTRODUCTION

There are at present a forty some antiviral drugs that have been formally licensed for clinical in the treatment of viral infections.³¹ These are mainly used in the treatment of infections caused by human immunodeficiency virus (HIV), hepatitis B virus (HBV), herpesviruses [herpes simplex virus (HSV), varicella-zoster virus (VZV),

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cytomegalovirus (CMV)], orthomyxoviruses (influenza), paramyxoviruses [respiratory syncytial virus (RSV)], and hepaciviruses [hepatitis C virus (HCV)]. As these are the viruses that are most in demand of antiviral therapy, they have prompted the search for new antiviral strategies and drugs directed towards either the same molecular targets as the approved antiviral drugs or to other targets.

Most of the newly described antiviral compounds (that are currently in development) are targeted at HIV, HBV or HCV. Some are targeted at HSV, VZV or CMV, but, there are, in addition, many other important viral pathogens for which medical intervention, either prophylactic or therapeutic, is highly needed, and, these are, among the DNA viruses, the papillomaviruses [human papilloma virus (HPV)], adenoviruses, poxviruses (variola, vaccinia, monkeypox, ...) and the herpesviruses Epstein–Barr (EBV) and human herpesvirus type 6 (HHV-6), and, among the RNA viruses, enteroviruses (i.e. Coxsackie B and Echo), coronaviruses [i.e. severe acute respiratory syndrome (SARS)-associated coronavirus], flaviviruses (i.e. Dengue, Yellow fever) and other RNA viruses associated with hemorrhagic fever [arenaviruses (i.e. Lassa fever), bunyaviruses (i.e. Rift Valley fever, Crimean-Congo fever) and filoviruses (i.e. Ebola and Marburg)].

Here I will describe, for each viral family, (i) which are the antiviral drugs that have been formally approved, (ii) which are the compounds that are under clinical development and thus may be considered as antiviral drug candidates, and (iii) which compounds are in the preclinical stage of development and still have a long route ahead before they could qualify as antiviral drugs (Table 1, Figure 1). The virus families to be addressed are the following: parvo-, polyoma-, papilloma-, adeno-, herpes-, pox-, picorna-, flavi-, corona-, orthomyxo-, paramyxo-, arena-, bunya-, rhabdo-, filo-, reo-, and retroviruses.

II. PICORNAVIRUSES (ENTERO- AND RHINOVIRUSES)

Among the enteroviruses, polio and hepatitis A can be efficiently controlled by vaccination: for polio both a life attenuated and an inactivated (“killed”) virus vaccine, whereas for hepatitis A an inactivated virus vaccine is available. The other enteroviruses (Coxsackie A and B and echoviruses) and the rhinoviruses need to be approached by chemotherapeutic agents. No single antiviral drug has ever been licensed for clinical use against entero- or rhinovirus infections. The most extensively studied for its potential against enteroviruses has

been pleconaril. This compound binds to a hydrophobic pocket beneath the “canyon floor” of the VP1 capsid protein of picornaviruses,⁹³ thereby “freezing” the viral capsid and preventing its dissociation (uncoating) from the viral RNA genome. The clinical efficacy of pleconaril has been evaluated in experimentally induced enterovirus (Coxsackie A21) respiratory infections in adult volunteers¹⁴⁵ and, on a compassionate basis, against potentially life-threatening enterovirus infections.¹⁴² Pleconaril has also been shown to reduce the duration and severity of picornavirus-associated viral respiratory illnesses in adolescents and adults.^{66,67} Pleconaril would also shorten the course of enteroviral meningitis (as compared to placebo recipients), albeit only modestly in patients with more severe disease.³⁷

For the prevention and/or treatment of rhinovirus infections (“common colds”) inhibitors of the human rhinovirus (HRV) 3C protease have been extensively investigated. Rupintrivir is an irreversible 3C protease inhibitor,⁴³ which, upon intranasal administration in human volunteers, appeared to be safe and well tolerated.⁷⁵ In experimentally induced rhinovirus colds in healthy volunteers, rupintrivir prophylaxis reduced the proportion of subjects with positive viral cultures but did not decrease the frequency of colds.⁶⁸ Another, irreversible inhibitor of HRV 3C protease, here referred to as a pyrrolidinyl pentenoic acid ethyl ester [compound 3⁴⁴ or compound 1¹³⁰] offers the advantage to be orally bioavailable: in healthy volunteers, single oral doses of this compound appeared to be safe and well tolerated, although the compound is currently not progressing toward clinical development.¹³⁰

Despite extensive research efforts that have led to the discovery of many potent antiviral agents, no drug today has been approved for the treatment or prevention of rhinovirus-associated illnesses. There are several reasons to consider when trying to understand this situation.¹²⁹ In the majority of individuals, rhinovirus-induced colds are mild and self-limiting. This alone dictates that potential drugs must be very safe and have a high risk/benefit ratio. The agent must have limited side effects and have no or low risk of resistance development and, furthermore, must be administered with low frequency (e.g. less than three times a day). To date no agent has been able to achieve these criteria and demonstrate appropriate clinical efficacy.¹²⁹

In great need of antiviral treatment are the often severe complications of Coxsackie B virus infections, such as myocarditis which may lead to idiopathic dilated cardiomyopathy. In mice, Coxsackie B3 virus-induced myocarditis is inhibited by the immunosuppressive agent mycophenolic acid (MPA) mofetil.¹²⁴ This beneficial outcome must apparently result from the immunosuppressive effect of MPA

(through inhibition of IMP dehydrogenase and, hence, GTP supply), since MPA did not reduce the infectious virus titers in the myocard. A more pronounced inhibitory effect on Coxsackie B3 virus-induced myocarditis, accompanied by a marked reduction in the virus titers in the heart, was obtained with the interferon inducer poly(I)·poly(C) and poly(I)·poly(C₁₂U) (also known as Ampligen), and to a lesser extent with (pegylated) interferon- α 2b.¹²⁵ Combination of an inhibitor of viral replication (such as Ampligen) with an immunosuppressant (such as MPA mofetil) could be an ideal treatment strategy for viral myocarditis, whether due to Coxsackie B or other viruses. How to implement such as treatment regimen in the clinical setting should be further addressed.

To investigate whether RNA interference (RNAi) can protect against Coxsackie virus B3 infection, several Coxsackievirus B3-specific small interfering RNAs (siRNAs) targeting distinct regions of the viral genome were evaluated, the most effective one (in inhibiting virus replication) being that targeting the viral protease 2A.²⁰⁰ A primordial requirement for being effective was a perfect sequence match in the central region of the target.²⁰⁰ As shown for poliovirus,⁵⁴ the virus may readily escape from RNA interference (RNAi) through unique point mutations (i.e. resulting in G:U mismatches) within the targeted regions; however, the emergence of resistant virus could be prevented by using a pool of siRNAs to simultaneously target multiple sites in the viral genome.⁵⁴

III. ALPHA- AND FLAVIVIRUSES (YELLOW FEVER, DENGUE, WEST NILE, ...)

No antivirals are currently available for the treatment of alpha- or flavivirus infections (although there is a live virus vaccine routinely used for the prophylaxis of Yellow fever), and the prospects for an effective therapy of flavivirus infections do not seem encouraging.⁹⁶ Antiviral compounds such as ribavirin have only weak activity against flaviviruses. Greater hope may be vested in interferon and interferon inducers. Based on infection of hamsters with the murine Modoc virus, an experimental flavivirus encephalitis model has been developed, which is reminiscent of Japanese encephalitis virus infection in humans.⁹⁷ In a related model with Modoc virus in SCID mice, both interferon- α 2b (whether pegylated or not) and interferon inducers [poly(I)·poly(C) and Ampligen] were shown to significantly delay virus-induced morbidity (paralysis) and mortality (due to progressive

encephalitis).⁹⁸ Ribavirin did not provide any beneficial effect in this model, whether given alone or in combination with interferon.

Recently, new inhibitors of flavivirus infection have been identified through high-throughput screening of a compound library. In particular, triaryl pyrazoline {[5-(4-chloro-phenyl)-3-thiophen-2-yl-4,5-dihydro-pyrazol-1-yl]-phenyl-methanone} was found to inhibit the *in vitro* replication of a number of flaviviruses (i.e. West Nile, Dengue, Yellow fever, St. Louis encephalitis) as well as some other viruses such as Western equine encephalitis virus (an alphavirus), mouse hepatitis virus (a coronavirus) and vesicular stomatitis virus (a rhabdovirus).¹³⁴ The triaryl pyrazoline would be specifically targeted at viral RNA synthesis.¹³⁴

RNA interference (RNAi) as demonstrated for hepadna-, picorna-, hepaci-, corona-, myxo- and retroviruses may also be further explored as an antiviral approach to control alpha- and flaviviruses, for example, at the level of their vector mosquitoes. RNA interference acts as a natural antiviral response to infection of *Anopheles gambiae* by the alphavirus O'nyong-O'nyong⁸² and for two mosquito-borne viruses, Semliki Forest virus (SFV) and Dengue virus (serotype 1), it has been shown that their replication in mosquito cells could be specifically blocked by si (double-stranded) RNA.¹³ This indicates that the use of specific siRNAs may inhibit virus replication in the insect host and thus prevent disease transmission.

IV. HEPACIVIRUSES (HCV)

Current, approved therapy for chronic hepatitis C consists of pegylated interferon- α 2 (180 μ g, parenterally, once weekly) combined with ribavirin (1000 or 1200 orally, daily).^{49,112} This treatment regimen is associated with a sustained viral response in at least 50% of the patients infected with HCV genotype 1, and of 80% in patients infected with another genotype (2, 3 or 4) of HCV. Duration of treatment is 48 weeks (or longer) for patients infected with HCV genotype 1, but may be reduced to 24 weeks for patients infected with another genotype.

Although interferon is generally acting as an immunomodulatory agent (i.e. in the treatment of hepatitis B) and ribavirin is an antiviral agent, when the two agents are used in combination against hepatitis C, they appear to act the other way around.³¹ Interferon appears to be targeted at the phosphoprotein encoded by the non-structural NS5A gene of the HCV genome.¹⁶⁶ thus achieving its antiviral effect, whereas

ribavirin, akin to MPA, primarily acts as an inhibitor of IMP dehydrogenase, thus reducing the biosynthesis of GTP. Ribavirin has recently been shown to modulate T-cell reactivity to HCV, i.e., by suppressing IL-10 production.¹⁴⁰

The combination of peginterferon α -2a with ribavirin has also been advocated for the therapy of HCV infection in patients with HIV coinfection.¹⁷⁸ However, it should not be forgotten that, should these patients be treated (for their HIV infection) with azidothymidine (zidovudine, ZDV), the latter may be antagonized by ribavirin.¹⁸⁰ Therefore it was re-assuring to note that ribavirin (at 800 mg/day) administered in combination with peginterferon α -2a did not significantly affect the intracellular phosphorylation or plasma pharmacokinetics of ZDV (or other pyrimidine dideoxynucleosides such as 3TC or d4T) in HIV/HCV-co-infected patients.¹⁴¹

In addition to peginterferon α -2a and peginterferon α -2b, other interferons are in clinical development, e.g. albuferon- α (IFN- α -2b fused to human serum albumin) which allows dosing at intervals of 2–4 weeks compared with one week for the peginterferons.³⁰ Consensus interferon (i.e. alfacon-1), when combined with ribavirin, has been shown to achieve a higher sustained response rate in naïve patients with chronic hepatitis C as compared to standard IFN- α and ribavirin.³⁰ Recently, a novel IFN- α variant (GEA 007.1) has been described which would have a better inhibitory activity than the standard IFN- α 2b in the HCV replicon system, due to a more potent activation of the JAK-STAT signaling pathway.⁴⁸

In the combination with peginterferon, ribavirin may be advantageously replaced by viramidine (taribavirin), its amidine analogue (which is converted, mainly in hepatocytes, by adenosine deaminase, to ribavirin), as the latter has a reduced uptake by, and, therefore, lesser toxicity for red blood cells, as compared to ribavirin.¹⁸⁶ Viramidine would give less anemia as compared with ribavirin. Phase III studies with viramidine combined with peginterferon, as compared to ribavirin combined with peginterferon, are eagerly awaited to assess which one to choose, ribavirin or viramidine.

Taking into account the duration of the combined interferon plus ribavirin treatment, the therewith associated side effects and costs, and the partial responses observed with this treatment regimen, fierce attempts have been made, rightfully, to develop more selective anti-HCV agents, targeted at specific viral proteins such as the NS3.4A serine protease and RNA helicase, and the NS5B RNA replicase (RNA-dependent RNA polymerase). Also the HCV p7 protein, which forms an

ion channel and can be blocked by long-alkyl-chain iminosugar derivatives, has been considered as a potential target for antiviral therapy.¹³¹

Proof-of-principle that compounds targeted at the NS3.4A protease could reduce plasma concentrations of HCV RNA has already been delivered with BILN 2061 administered orally for no longer than 2 days in patients infected with HCV genotype 1.⁸⁹ BILN 2061 (Ciluprevir), for which an efficient large-scale synthetic procedure has been described recently,¹⁹⁶ was able to reduce HCV RNA levels by 2–3 log₁₀ in patients infected with HCV genotype 1, after 2 days of treatment,⁷² but in patients infected with HCV genotypes 2 or 3, it proved less effective, apparently due to a lower affinity of BILN 2061 for the HCV protease of genotypes 2 and 3, as compared to genotype 1.¹³⁸ Replacement of five residues at positions 78, 79, 80, 122, and 132 could account for most of the reduced sensitivity of genotype 2b protease,¹⁶⁹ while replacement of residue 168 alone,¹⁶⁹ or in combination with substitution of residues at positions 123 and 132,¹⁷⁷ could account for the reduced sensitivity of genotype 3a. Apparently, the rigidity of BILN 2061, while conferring greater potency against genotype 1, rendered it more sensitive to variations near its binding site at the NS3.4A protease.¹⁷⁷ Despite the robust antiviral response observed with BILN 2061 in genotype 1 HCV-infected individuals, further clinical development of this compound was halted because of (cardio)toxicity issues in animals.

Another NS3.4A protease inhibitor, which differs in its *in vitro* resistance profile from BILN 2061, is VX-950 (telaprevir).¹⁰² While substitution of Ala156 with either valine (A156V) or threonine (A156T) led to cross-resistance between BILN 2061 and VX-950 [but also reduced fitness (or replication capacity) in a transient replicon cell system,¹⁰³ the major BILN 2061-resistant mutants (D168V and D168A) were fully susceptible to VX-950, and, *vice versa*, the dominant VX-950-resistant mutant (A156S) remained sensitive to BILN 2061.^{102,103} Thus, VX-950 and BILN 2061 must elicit resistance to HCV protease (NS3.4A) by different mechanisms.¹⁰³

VX-950 (750 mg every 8 hours) was found to achieve, at the end of a 14 day-treatment, a main reduction of HCV RNA of 4.4 log₁₀. Out of all patients receiving VX-950, 26/28 showed a >3 log decline of HCV RNA.¹³⁷ [In some patients dosed with VX-950, the virus became undetectable at day 14 of dosing.] The overall preclinical profile of VX-950 supports its candidacy as a novel oral therapy against hepatitis C.¹³² The combination of VX-950 and interferon- α was found to be additive to synergistic in reducing HCV RNA in replicon cells, and this combination also suppressed the emergence of *in vitro* resistance mutations

against VX-950 in replicon cells.¹⁰⁴ Following promising results with VX-950 in phase I clinical trials, the compound has now progressed to phase II trials where it is evaluated in combination with peginterferon- α with or without ribavirin.

In addition to VX-950, other, 7-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid-based macrocyclic inhibitors of HCV NS3.4A protease²⁰ as well as SCH 503034, a mechanism-based inhibitor of HCV NS3.4A protease,^{179,109} are in preclinical development. In fact, the latter was found to act synergistically with α -interferon in suppressing HCV replicon synthesis.¹⁰⁹ SCH 503034 [compound **70** in Venkatraman et al.¹⁷⁹] demonstrated good oral bioavailability in rats and dogs¹⁷⁹ and has been advanced to clinical trials in humans for the treatment of HCV infections. SCH 503034 at the highest dose used 400 mg three times daily for 14 days achieved a 1.5 log₁₀ decline in viremia²⁰¹ SCH 503034 is now being evaluated in combination with pegylated interferon- α , with or without ribavirin in ongoing 48-week phase II studies.

The HCV protease inhibitor SCH 503034 selected for a number of mutations, i.e. T54A, V170A, A156S and A156T; the A156T mutation conferred the highest level of resistance to SCH 503034 but also led to the greatest reduction in fitness.¹⁷⁶ The A156T mutation also conferred high level resistance to SCH6 (SCH 446211), a novel ketoamide (structurally related to SCH 503034) inhibitor of the NS3.4A protease.¹⁹⁸ A novel mutation, R109K, was identified which conferred moderate resistance only to SCH6. Unlike R109K which had minimal impact on NS3.4A enzymatic activity, A156T significantly reduced the enzymatic activity, polyprotein processing and replication fitness.¹⁹⁸ However, three separate second-site mutations, P89L, Q86R and G162R were capable of partially reversing A156T-associated fitness without significantly reducing resistance to the protease inhibitor.¹⁹⁸

In addition to the NS3.4A protease, the NS5B RNA replicase has also been perceived as an attractive target for the development of HCV inhibitors. Highly potent and selective antiviral agents (i.e. VP32947) [*N*-propyl-*N*-[2-(2*H*-1,2,4-triazino[5,6-*b*]indol-3-ylthio)ethyl]-1-propanamine]⁵ and compound 1453 [1-[2-diethylamino)ethyl]-6-(1*H*-imidazol-1-yl)-1,3-dihydro-2*H*-benzimidazol-2-one]¹⁶⁵ targeted at the viral RNA replicase have been described to inhibit the replication of bovine viral diarrhoea virus (BVDV), a pestivirus which could be considered as a surrogate virus for HCV^{5,165} We have recently described two novel series of compounds [prototypes: 5-[(4-bromophenyl)methyl]-2-phenyl-5*H*-imidazo[4,5-*c*]pyridine (BPIP)]¹²⁶ and ethyl-2-methylimidazo[1,2-*a*]pyrrolo[2,3-*c*]pyridin-8-carboxylate AG110,¹²⁸ which act as “non-

nucleoside" RNA replicase inhibitors (NNRRIs) and effect a highly potent and selective inhibition of the replication of BVDV.^{126,128} From the BPIP class of compounds,¹³⁵ new congeners have been derived that act equally efficiently against HCV replication.¹³⁶ In future treatment strategies for HCV infections, these NNRRIs may likely to be combined with "nucleoside" RNA replicase inhibitors (NRRIs), in analogy with the strategy followed for the treatment of HIV infections, where NRTIs are combined with NNRTIs (see *infra*).

The sole NRI which has already proceeded to phase I/II clinical trials for the therapy of hepatitis C is the 3'-O-valine ester of 2'-C-methylcytidine (NM-283, valopicitabine)I, which can be administered by the oral route^{33,2} and shows enhanced antiviral efficacy if combined with peginterferon (valopicitabine is currently in phase II combination trials with pegylated interferon- α). It would be wise not to add ribavirin to this combination, as it has been shown that ribavirin antagonizes the *in vitro* anti-HCV activity of 2'-C-methylcytidine, the active compound of valopicitabine.²⁸

HCV replicons that are resistant to 2'-C-methylcytidine (and 2'-C-methylpurine nucleosides) can be readily isolated *in vitro*, and resistance is the result of the S282T mutation in the NS5B gene. As a consequence of the S282T mutation, 2'-C-methyl CTP is no longer incorporated during the initiation step of RNA synthesis. In addition, the presence of the S282T mutation also compromises incorporation of the natural nucleotides, which may translate in decreased viral fitness.⁴⁵

In addition to 2'-C-methylcytidine, several other ribonucleoside analogues (NRRIs) have been reported to inhibit HCV replication (as reviewed by):¹⁷⁴ 2'-O-methylcytidine,¹⁴ 2'-C-methyladenosine,^{14,171} 7-deaza-2'-C-methyladenosine,¹²² 2'-C-methylguanosine,^{115,46} 2'-deoxy-2'-fluoro-2'-C-methylcytidine (PSI-6130),¹⁵⁹ and 4'-azidocytidine (R1479).⁸⁶

PSI-6130 is a specific inhibitor of HCV.¹⁵⁹ It has no anti-BVDV activity. The fact that PSI-6130 shows reduced activity against the RdRp S282T mutant, which is resistant to 2'-C-methylated nucleosides suggests that PSI-6130 is an inhibitor of HCV RNA synthesis.¹⁵⁹ In contrast, R1479 (4'-azidocytidine) did not show cross-resistance with 2'-C-methylcytidine.⁹¹ Thus, the S282T mutation⁸⁶ did not confer resistance to R1479. *In vitro* studies mapped resistance to R1479 to amino acid substitutions S96T and S96T/N142T of the NS5B polymerase.⁹¹

All the aforementioned nucleoside analogues are, in principle, non-obligate chain-terminating nucleoside analogues. It would now seem interesting to prepare and evaluate the corresponding obligatory chain-terminating 3'-deoxyribonucleoside analogues for their anti-

HCV activity. This was recently done for 2'-C-methylcytidine and 2'-C-methyluridine, which were modified at the C-3' position.¹³³ The newly synthesized compounds were quoted as antivirally inactive, but HCV did not figure among the viruses that were evaluated for their susceptibility to the compounds.

A new class of HCV NS5B RNA replicase inhibitors is represented by the α , γ -diketo acids (DKAs),^{162,163} one of the more active DKAs being DKA compound 30. These compounds are reminiscent of the DKA type of HIV integrase inhibitors (see *infra*) and are assumed to inhibit the HCV polymerase activity *via* chelation of the active site Mg^{++} ions. In a certain sense they may be considered as "pyrophosphate mimics", thus acting as product-like inhibitors of the polymerase reaction.¹⁷⁴

In recent years, a pleiade of NNRRIs has been described to act in a very similar ("allosteric") fashion with HCV NS5B RNA replicase as NNRTIs do with respect to the HIV reverse transcriptase: benzimidazole-based derivatives¹⁷² [i.e., benzimidazole 5-carboxamide derivatives,^{7,88} indole-*N*-acetamide derivatives,^{61,39} benzo-1,2,4-thiadiazine derivatives,^{38,173} phenylalanine derivatives,¹⁸³ thiophene 2-carboxylic acid derivatives,^{17,8} dihydropyranone derivatives,¹⁰⁷ the tetrahydropyranoindolyl acetic acid derivative HCV-371 (R-enantiomer of the racemic mixture HCV-570)^{73,74} and the *N*-1-aza-4-hydroxyquinolone benzothiadiazine A-782759.¹¹⁷ The allosteric binding site for some of these compounds have already been identified by crystallographic studies.^{39,183,17,8} It corresponds to a narrow cleft on the protein's surface in the "thumb" domain, about 30–35 Å from the enzyme's catalytic center.^{183,17}

Curiously, most of the NNRRIs that are active against the HCV NS5B polymerase contain, besides a large hydrophobic region, a carboxylic acid group (or a similar motif) that allows hydrogen bonding with main chain amide nitrogen atoms (i.e. Ser 476 and Tyr 477, as demonstrated for the phenylalanine derivative).¹⁸³ For the indole-*N*-acetamide derivatives, it has been suggested that they may displace part of the fingertip loop anchoring the fingers domain to the thumb domain.³⁹ Using the thiophene 2-carboxylate type of inhibitors, it has been reported that these inhibitors can only be soaked in crystal form I (which adopts a "closed" conformation that is believed to be the active form, and not in form II (which adopts an "open" conformation, and is thus in the inactive form).⁸ Resistant mutations that emerged with the benzimidazole 5-carboxamide and related compounds were found at three amino acid positions in the thumb domain: Pro495 (P495S/L/A/T), Pro496 (P496S/A) and Val499 (V499A); mutation at

each of these positions conferred different levels of resistance (in decreasing order P495S/L/A/T > P496S/A > 499A).⁸⁸

Mutations conferring resistance to both the HCV NS5B RNA replicase (i.e. H95Q, N411S, M414L, M414T or Y448H) and NS3 protease (i.e. A156V or D168V) have been identified).¹¹⁷ These mutations conferred high levels of resistance to A-782759 and BILN 2061, respectively. However, the A-782759-resistant mutants remained susceptible to the NRRIs and other classes of NNRRIs, as well as interferon. In addition, the dually (A-782759- and BILN 2061-) resistant mutants displayed significantly reduced replicative ability as compared to the wild-type. These findings support a rationale for drug combinations in the therapy of HCV infections.¹¹⁷

As recently reviewed,³⁰ several other approaches, including ribozymes, antisense oligonucleotides, and RNA interference (RNAi) based on short interfering (si)RNAs could be envisaged to target the HCV genome. ISIS 14803, a 20-base antisense oligonucleotide complementary to the internal ribosome entry site (IRES), in a phase I clinical trial gave transient HCV reduction (1.2–1.7 log₁₀) in 3/28 patients but ALT (alanine aminotransferase) flares up to 10-fold in 5/28 patients.¹¹³

Short interfering (si)RNAs, aimed at posttranscriptional gene silencing, may be considered an attractive approach to curtail HCV infections.⁸⁷ The siRNA approach has proven to be efficacious *in vivo*, in suppressing (SARS) coronavirus infections¹⁰⁰ and influenza A virus infections^{52,175} in monkeys¹⁰⁰ and mice,^{52,175} respectively.

From the screening of various marketed compounds, arsenic trioxide (As₂O₃) emerged as a potent HCV inhibitor.⁷⁷ Similarly, sodium stibogluconate (SSG), along with several other antimonial compounds, including Sb₂O₃ and SbCl₃ were found to exert potent anti-HCV activity at concentrations that did not affect cell viability.⁷⁸ When SSG was combined with interferon- α , these two drugs acted synergistically to suppress HCV replication.⁷⁸

Various cellular targets may also be envisaged to interfere with HCV infections. In this sense, α -glucosidase inhibitors such as deoxynojirimycin have been shown to disturb the morphogenesis of HCV-like particles and may eventually be useful to fight HCV infections as part of drug combination protocols.¹⁸ Another cellular target for chemotherapeutic intervention is cyclophilin B (CyP B) which is critical for the efficient replication of the HCV genome. CyP B is a functional regulator of the HCV RNA polymerase.¹⁸⁵ Cyclosporin A which interacts with CyP B inhibits HCV replication *in vitro*.¹⁸⁵ We have recently demonstrated that the non-immunosuppressive cyclosporin DEBIO-025 is even 10-fold more potent an inhibitor of HCV replication.¹²⁷

DEBIO-025 proved also additive to slightly synergistic when combined with interferon- α 2a, and at concentrations of 0.5 and 1 $\mu\text{g/ml}$ was able to clear the cells from their HCV replicon within three to four passages, whereas treatment with cyclosporin A at the same concentrations for seven consecutive passages did not result in clearance of the HCV replicon.¹²⁷ DEBIO-025 may form an attractive new option for the therapy of HCV infections, particularly in HCV/HIV co-infected patients,¹²⁷ and the clinical studies with DEBIO-025 have been recently initiated.

V. CORONAVIRUSES (SARS)

As for HCV, there are several proteins encoded by the SARS coronavirus which could be considered as targets for chemotherapeutic intervention: i.e. the spike (S) protein, the 3C-like main protease, the NT-Pase/helicase, the RNA-dependent RNA polymerase (RNA replicase), and, possibly, other viral (or cellular) protein-mediated processes.¹⁵⁷ The SARS coronavirus S protein mediates infection of permissive cells through interaction with its receptor, the angiotensin-converting enzyme 2 (ACE 2),¹⁰¹ and monoclonal antibody to the S1 domain was found to neutralize the virus by blocking its association with the receptor ACE 2.¹⁶¹

Also the fusion of the SARS coronavirus with the cell could be considered an attractive target. To the extent that this fusion process bears resemblance to the fusogenic mechanism of HIV, i.e. with regard to heptad repeat interactions and six-helix bundle formation, it might be feasible to develop SARS coronavirus inhibitors, analogous to the HIV fusion inhibitor enfuvirtide.¹⁰⁵

Following receptor binding and induced conformational changes in the spike glycoprotein, a third step would be involved in the viral entry process, namely cathepsin L proteolysis within endosomes.¹⁵⁴ The cathepsin-L-specific inhibitor, MDL 28170 [also known as calpain inhibitor III, or Z-Val-Phe(CHO)], at the same time inhibited cathepsin-L activity and S protein-mediated infection (at an IC_{50} of 2.5 nM and 0.1 μM , respectively). In addition to calpain inhibitor III, some other calpain inhibitors have been described as inhibitors of SARS coronavirus replication, the most selective (selectivity index >100) being calpain inhibitor VI [4-fluorophenylsulfonyl-Val-Leu(CHO)].⁶

The crystal structure of the SARS coronavirus protease has been revealed.^{194,94} This offers a solid basis for the rational drug design of SARS protease inhibitors. For other potential targets such as the

NTPase/helicase and the RNA replicase (RNA-dependent RNA polymerase) such structural basis still has to be delineated.

Of a number of peptidomimetic compounds (aziridinyl peptides, ketoglutamine analogues, chymotrypsin-like protease inhibitors and peptide anilides) that have been reported as inhibitors of the SARS coronavirus main protease, the niclosamide anilide, with a $K_i = 0.03 \mu\text{M}$ ($\text{IC}_{50} = 0.06 \mu\text{M}$), proved to be the most potent (competitive) inhibitor.¹⁴⁶ There are only a few cases where the 3C-like protease inhibitors were shown to inhibit both the SARS coronavirus protease activity and virus replication in cell culture. For example, the Phe–Phe dipeptide inhibitor was found to inhibit the 3C-like protease at an IC_{50} of $1 \mu\text{M}$ and inhibited virus replication in Vero cells at an EC_{50} of $0.18 \mu\text{M}$, while not being toxic to the host cells at a concentration of $200 \mu\text{M}$ (selectivity index: >1000).¹⁴⁷

Recently, TG-0205221 was described as a potent SARS coronavirus 3C-like protease inhibitor ($K_i = 53 \text{ nM}$), which reduced virus production in cell culture by $4.7 \log_{10}$ at a concentration of $5 \mu\text{M}$.¹⁹⁵ An octapeptide, specifically designed for the SARS coronavirus main protease, namely AVLQSGFR, was reported to inhibit SARS coronavirus replication in Vero cells at an EC_{50} of $0.027 \mu\text{g/ml}$, while not being cytotoxic at $100 \mu\text{g/ml}$, thus establishing a selectivity index of > 3700 .⁵¹ Whether this highly selective antiviral effect was actually mediated by an inhibition of the SARS coronavirus main protease was not ascertained in this study.⁵¹

The SARS coronavirus NTPase/helicase has been considered a potential target for the development of anti-SARS agents.¹⁶⁷ Bananin and three of its derivatives (iodobananin, vanillinbananin and eubananin) were shown to inhibit both the ATPase and helicase activity of the SARS coronavirus NTPase/helicase, with IC_{50} values (for the ATPase activity) in the range of $0.5\text{--}3 \mu\text{M}$.¹⁶⁸ Bananin was also found to inhibit SARS-CoV replication in fetal rhesus kidney (FRhK-4) cells at an EC_{50} of less than $10 \mu\text{M}$ and a CC_{50} of over $300 \mu\text{M}$, thus exhibiting a selectivity index of over 30.¹⁶⁸ Whether the antiviral effect obtained in cell culture was causally linked to inhibition of the NTPase/helicase was not ascertained.

The SARS coronavirus RNA-dependent RNA polymerase (RdRp), because of its pivotal role in viral replication, represents another potential target for anti-SARS therapy. This enzyme does not contain a hydrophobic pocket for non-nucleoside inhibitors similar to those that have proven effective against the HCV polymerase or HIV-1 reverse transcriptase.¹⁹² In fact, non-nucleoside HIV-1 reverse transcriptase inhibitors were shown to have no evident inhibitory effect on SARS

coronavirus RdRp activity).²¹ At present, few, if any, nucleoside analogues have been recognized as specific inhibitors of the SARS coronavirus RdRp. There is N⁴-hydroxycytidine, which has been accredited with both anti-HCV and anti-SARS coronavirus effects. Against SARS coronavirus it proved active at an EC₅₀ of 10 μ M (selectivity index ≥ 10)⁶ Whether this antiviral effect was mediated by an inhibition of the viral RdRp was not ascertained, however.

A wide variety of “old” and “new” compounds have been reported to inhibit the *in vitro* replication of the SARS coronavirus at relatively high concentration (≥ 1 μ M).³¹ There is no shortage of small molecules that inhibit the replication of the SARS virus within the 1–10 μ M (or higher) concentration range,¹⁸⁸ but whether any of these molecules would be able to prevent or suppress SARS *in vivo*, remains to be determined. Typical examples of such miscellaneous compounds, often with an ill-defined mode of action but selectivity indexes up to 100, that have been reported to inhibit SARS coronavirus replication are valinomycin,¹⁸⁸ glycyrrhizin,²⁵ chloroquine,⁸³ niclosamide¹⁸⁹ and nelfinavir.¹⁹³

Short interfering (si) RNAs have been developed that target the replicase⁷⁰ and spike (S)²⁰² genes of the SARS coronavirus genome, thereby silencing their expression in cell culture. Potent siRNA inhibitors of SARS coronavirus *in vitro* [i.e. the siRNA duplexes siSC2 (forward sequence: 5'-GCUCCUAAUACACUCAACdtdt-3') and siSC5 (forward sequence: 5'-GGAUGAGGAAGGCAAUUUAdtdt-3'), targeting the SARS coronavirus genome at S protein- and non-structural protein-12-coding regions, respectively] were further evaluated for their efficacy in a rhesus macaque SARS model,¹⁰⁰ and found to provide relief from SARS coronavirus infection-induced fever, diminish SARS-CoV levels and reduce acute diffuse alveolar damage. Further studies with siRNA in prophylactic and therapeutic regimens against SARS coronavirus seem warranted.

Shortly after SARS coronavirus was identified as the causative agent of SARS, interferons were shown to inhibit the replication of SARS coronavirus in cell culture *in vitro*, interferon- β being more potent than either interferon- α or - γ .²⁶ These observations were subsequently confirmed and extended in several other studies.^{71,158,143} Interferon- β , in conjunction with interferon- γ , was found to synergistically inhibit the replication of SARS coronavirus in Vero cells.¹⁴³ Being a prophylactic rather than therapeutic agent, interferon(s) may have their highest utility in the prophylaxis or early post-exposure management of SARS. Pegylated interferon- α has been shown to reduce viral replication and excretion, viral antigen expression by type 1 pneumocytes and the

attendant pulmonary damage in cynomolgous macaques that were infected experimentally with SARS coronavirus.⁵⁸ Pegylated interferon- α is commercially available for the treatment of hepatitis C (where it is generally used in combination with ribavirin) and hepatitis B. Pegylated interferon- α as well as the other commercially available interferons (interferon- β , alfacon-1, etc.) could be considered for prevention and/or early post-exposure treatment of SARS should it re-emerge.

VI. ORTHOMYXOVIRUSES (INFLUENZA A, B)

For many years, amantadine and rimantadine have been used for the prophylaxis and therapy of influenza A virus infections, but they never gained wide acceptance, primarily because of the risk of rapid emergence of drug-resistant virus mutants. These compounds interact specifically with the matrix M2 protein, which through its function as an hydrogen ion (H^+) channel, helps in the decapsulation (“uncoating”) of the influenza A virus particles. Influenza A (H1N1) viruses harboring amantadine-resistance mutations are as virulent as wild-type virus strains¹ and can be readily transmitted during antiviral pressure in the clinical setting.

In recent years, the neuraminidase inhibitors zanamivir¹⁸¹ and oseltamivir⁸⁴ have become available for the therapy and/or prophylaxis of influenza A and B virus infections. Influenza has adopted a unique replication strategy by using one of its surface glycoproteins, hemagglutinin (H), to bind to the target cell receptor [which contains a terminal sialic acid (*N*-acetylneuraminic acid, NANA)], and another surface glycoprotein neuraminidase (N), to cleave off the terminal sialic acid, thus allowing the virus particles to leave the cells after the viral replicative cycle has been completed³¹ Neuraminidase inhibitors block the release of progeny virus particles from the virus-infected cells, thus preventing virus spread to other host cells.

When used therapeutically, neuraminidase inhibitors lead to a reduction in illness by 1–2 days, a reduction in virus transmission to household or healthcare contacts, a reduction in the frequency and severity of complications (such as sinusitis and bronchitis) and a diminished use of antibiotics.⁸¹ When used prophylactically, neuraminidase inhibitors significantly reduced the number of new influenza cases.⁶⁹ Although resistance of human influenza viruses to neuraminidase inhibitors can develop,⁸⁵ there is no evidence of naturally occurring resistance to either zanamivir or oseltamivir.^{114,118} Zanamivir and oseltamivir should be effective against both influenza

A and B, and, among influenza A, the prevailing variants H₁N₁, H₃N₂ and, also, the avian “flu” H₅N₁. Zanamivir, which must be taken through (oral) inhalation, and, in particular, oseltamivir, which can be more conveniently administered as oral capsules, should be stockpiled to affront a potential influenza pandemic in the future.^{184,123} With the increasing threat of the avian “flu” (H₅N₁), the need for a sufficient supply of neuraminidase inhibitors, such as oseltamivir and zanamivir, has become extremely urgent. In comparison with the neuraminidase inhibitors, the existing influenza vaccines are likely to be of limited value against newly emerging influenza virus strains.

Following zanamivir and oseltamivir, similar structure-based neuraminidase inhibitors have been developed, such as the cyclopentane derivative RWJ-270201 (peramivir)^{155,152} and the pyrrolidines A-192558¹⁸² and A-315675.^{36,60} These novel neuraminidase inhibitors may themselves be considered as potential drug candidates, and, while being amenable to further optimization,¹¹⁰ lead to the development of yet newer compounds with improved activity, bioavailability and/or resistance profiles.

In fact, both peramivir (RWJ-270201) and A-315675 proved effective against a panel of five zanamivir-resistant and six oseltamivir-resistant A and B influenza virus strains.¹¹⁶ Oseltamivir resistance in clinical isolates of human influenza A has been associated with mutations at positions 119, 198, 274, 292 or 294 of the neuraminidase. Recently, resistance of avian influenza A H₅N₁ against oseltamivir was shown to be caused by the H274Y mutation.^{92,34} The H274Y variant still appeared sensitive to zanamivir.⁹² Prominent among the other neuraminidase inhibitor-resistant influenza A (H₃N₂) virus mutations (not yet demonstrated for H₅N₁) are E119V and R292K: whereas the R292K mutation was associated with compromised virus growth and transmissibility, the growth and transmissibility of the E119V variant were comparable to those of wild-type virus.¹⁹⁷

The emergence of antiviral drug resistance during oseltamivir treatment and its association with clinical failure in immunocompromised hosts⁷⁹ and influenza A H₅N₁-infected patients³⁴ has highlighted the need for alternative therapies and the use of antiviral combinations.⁶³ The concept of using two or more antivirals to enhance the antiviral effects and perhaps reduce resistance emergence is decades old.^{62,64,90}

Are there other antiviral agents, besides amantadine, rimantadine and the neuraminidase inhibitors, which may be considered for their potential, in the prevention and/or therapy, of influenza A virus infections, including avian influenza (H₅N₁)? Ribavirin has since long been recognized as a broad-spectrum antiviral agent, with particular

activity against both ortho- and paramyxoviruses.¹⁵¹ While earlier observations point to the lack of oral ribavirin (at 1 g daily) in naturally occurring influenza A (H1N1) virus infection,¹⁵⁶ intravenous ribavirin (at 5 mg/kg/hour for 8 hours, followed by 1.5 mg/kg/hour for 2 to 6 days) may be worth exploring as a therapeutic strategy for serious influenza and parainfluenza virus infections.⁶⁵

Recently, viramidine, the carboxamidine analogue of ribavirin was shown to have similar efficacy as ribavirin against influenza virus infections, and considering the lesser toxicity, viramidine may warrant further evaluation as a possible therapy for influenza, including H5N1.¹⁵³ Yet, other recently described compounds with specific activity against influenza A, B and C viruses are T-705 (6-fluoro-3-hydroxy-2-pyrazine carboxamide) and the 2,6-diketopiperazine flutimide, which would target the viral polymerase⁵⁰ and cap-dependent endonuclease,¹⁷⁰ respectively. In fact, the polymerase (PA, PB1, PB2) complex contributes to the high virulence of avian influenza H5N1, and has been considered an attractive target for novel anti-influenza virus drug development.¹⁴⁴

Other approaches to curtail a potential influenza virus epidemic include carbohydrate-binding molecules, such as the defensin retrocyclin 2, which inhibit viral fusion and entry by cross-linking membrane glycoproteins, and which have been shown to inhibit influenza A virus infection,⁹⁵ as well as T- and M-tropic HIV-1 virus infections.²⁹ Furthermore, as has been shown for several other virus infections, i.e. HCV infections⁸⁷ and (SARS) coronavirus infections,^{70,202,100} short interfering RNAs (siRNAs) specific for conserved influenza virus genes may also be expected, and have in fact been demonstrated, to protect animals against highly pathogenic avian influenza A viruses.^{52,175}

VII. PARAMYXOVIRUSES (PARAINFLUENZA, MEASLES, MUMPS, RSV, HMPV, NIPAH, ...)

Of the paramyxoviruses, parainfluenza (types 1–5) has received little attention from either a preventative or curative viewpoint; mumps and measles, like the rubellivirus rubella, are now considered to be sufficiently contained by vaccination (although, despite existence of a vaccine, over half a million of deaths per year result from measles virus, which has prompted the search for fusion inhibitors that block measles virus entry),¹⁶⁴ which makes RSV (respiratory syncytial virus) and hMPV (human metapneumovirus) as well as Nipah, the paramyxoviruses with the greatest need for antiviral therapy.

For RSV the only approved antiviral therapy is aerosol administration of ribavirin. In practice, however, ribavirin is rarely used owing to the technical burden of delivery by aerosol under the given circumstances (RSV bronchopneumonitis in young infants). Recently, intravenous ribavirin (together with oral corticosteroids) has proved to be a safe and cost-effective treatment for RSV infection after lung transplantation: RSV represents a risk factor for bronchiolitis obliterans syndrome, the major limiting factor for long-term survival after lung transplantation.⁵⁵

Given the high incidence of RSV infections (which are often diagnosed as influenza), there is a high (an as yet unmet) medical need for an appropriate therapy (and prophylaxis) of RSV infections; The same holds for hMPV infections, which usually occur during the same (winter) season as RSV, mainly in young children, elderly people and immunocompromised individuals. Ribavirin certainly holds promise for the treatment of hMPV infections, as has recently been demonstrated in the mouse model for hMPV.⁵⁹

As has been mentioned above for HCV,⁸⁷ short interfering RNAs (siRNAs) may also be applicable, if properly designed and administered (intranasally) in the treatment of respiratory virus infections:⁹ using the RNA interference (RNAi) approach, Bitko et al.⁹ demonstrated that respiratory syncytial virus and parainfluenza virus infections, both individual and joint, could be specifically prevented and inhibited by siRNAs instilled intranasally in the mouse, with or without transfection reagents. In the case of measles, however, the RNAi approach may either prevent or enhance viral transcription, depending on the target mRNA [nucleocapsid (N), phosphoprotein (P) and polymerase (L) mRNA *versus* the matrix (M) mRNA].¹³⁹ For avian metapneumovirus, one of the major causes of respiratory infections in poultry, siRNAs specific towards the phosphoprotein (P) led to marked inhibition of virus replication.¹¹⁹

Recently, a number of small molecules, i.e. VP-14637 and JNJ-2408068 (formerly known as R-170591), although structurally dissimilar, have been shown to fit into a small hydrophobic cavity in the inner core of the RSV fusion (F) protein, thereby interacting with the heptad repeats HR1 and HR2 domains, and to inhibit RSV fusion.^{41,42,4,190} Although the therapeutic potential of these compounds in the treatment of RSV infections is presently unclear, there is no doubt that further exploration of the mechanism of interaction between these inhibitors and the F protein should facilitate the design of new RSV fusion inhibitors.⁴² In cotton rats, treatment by VP-14637 (or JNJ-2408068)

small droplet aerosol for 60 min (or 15 min) significantly reduced mean lung virus titers.^{190,191}

BMS-433771 was found to be a potent inhibitor of RSV replication *in vitro*.²² it exhibited excellent potency against multiple laboratory and clinical isolates of both A and B RSV with an average EC₅₀ of 20 nM. BMS-433771 inhibits fusion the (viral and cellular) lipid membranes during both the early and virus entry stage and late-stage syncytium formation.²² BMS-433771 was shown to be orally active against RSV in BALB/c mice and cotton rats, even if administered as a single oral dose 1 hour prior to intranasal RSV inoculation.²³ It could be considered the prototype of small-molecular-weight inhibitors that target the formation of the six helical coiled-coil bundles as a prelude to virus-cell fusion, not only of RSV but also HIV.²⁴ In fact, starting from BMS-433771 new water-soluble (i.e. carboxylic acid-substituted benzimidazol-2-one derivatives have been synthesized with potent *in vitro* and *in vivo* activity against RSV.¹⁹⁹

Peptides containing multiple copies of alternating HR1 and HR2 sequences of the terminal heptal repeats of the F-protein have been designed to inhibit F-protein-mediated fusion.¹²⁰ MBX 300 or [2,2-bis(docosyloxymethyl)propyl-5-acetoamido-3,5-dideoxyl-4,7,8,9-tetra-O-(sodium-oxysulfonyl)-D-glycero-D-galacto-2-nonulopyranosid]-onate, on the other hand, inhibits RSV attachment to the cell by targeting the G-protein.⁴⁰

RSV attachment/fusion inhibitors targeted at either the G- or F-protein have, despite their marked *in vitro* potency and *in vivo* efficacy in the cotton rat model, not made much progress in the clinical setting, in part because of their poor pharmacokinetic properties. Therefore, new RSV inhibitors acting at a target distinct from the attachment/fusion process have been searched for. One such target is the viral nucleocapsid (N) protein which appears to be the target for the 1,4-benzodiazepines (i.e. A-60444).¹⁵ The compound has entered phase II clinical trials (according to),¹⁵⁰ but data have not yet been made available.

Another target protein worth pursuing for potential anti-RSV agents is the RNA-dependent RNA polymerase L-protein, which has been identified as the point of attack for 4-amino-8-(3-([2-(3,4-dimethoxyphenyl)ethyl]amino)propyl)-6,6-dimethyl-2-(4-methyl-3-nitrophenyl)-1*H*-imidazo[4,5-*h*]-isoquinoline-7,9(6*H*, 8*H*)-dione ("Compound D")¹⁰⁶ and 6-[4-[(biphenyl-2-ylcarbonyl)amino]benzoyl]-*N*-cyclopropyl-5,6-dihydro-4*H*-thieno[3,2-*d*][1]benzazepine-2-carboxamide (YM-53403).¹⁶⁰ Whether these compounds ("compound D" and YM-53403) have any

Table 1. The past, present and future of antiviral drugs (Part II: RNA viruses except retroviruses)

Virus	Compound		
	Approved for medical use	In clinical development	In preclinical evaluation
Picorna (Entero, Rhino)	–	Pleconaril Ruprintrivir	Pyrrolidinyl pentenoic acid (ethyl ester) Mycophenolic acid (MPA) mofetil Interferon (inducers)
Alpha and Flavi (Yellow fever, Dengue, West Nile, ...)	–	–	Triaryl pyrazoline Interferon (inducers)
Hepaci (HCV)	Pegylated interferon- α combined with ribavirin	BILN 2061 (Ciluprevir) VX-950 (Telaprevir) NM 283 (Valopicitabine) Viramidine (Taribavirin) SCH 503034 DEBIO-025	SCH 446211 (SCH6) 2'-C-methylcytidine 2'-O-methylcytidine 2'-C-methyladenosine 7-Deaza-2'-C-methyladenosine 2'-C-methylguanosine 2'-Deoxy-2'-fluoro-2'-C-methylcytidine 4'-Azidocytidine (R1479) DKA compd 30 Benzimidazole derivative Benzimidazole 5-carboxamide derivative

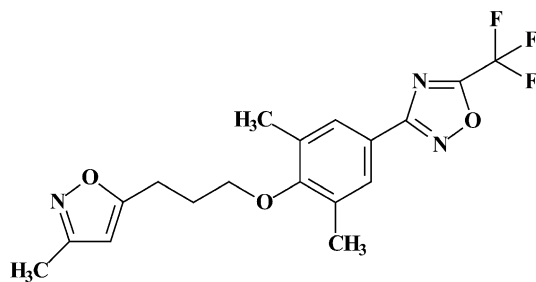
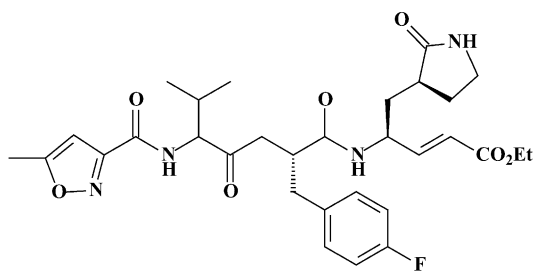
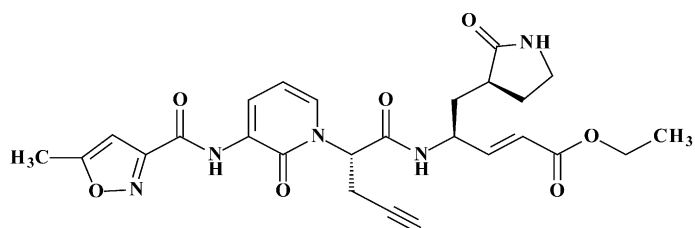
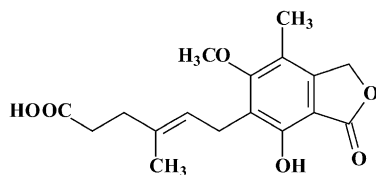
Table 1. — Continued

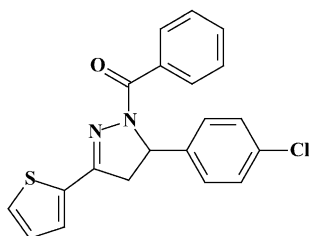
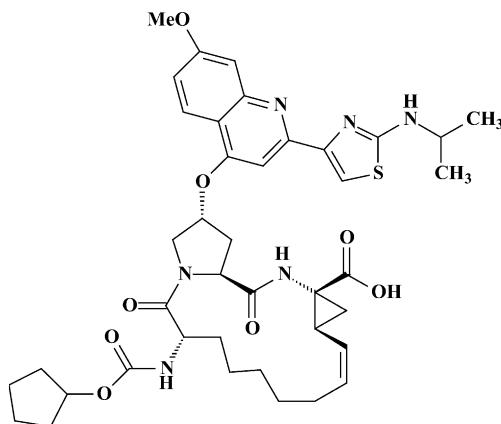
Virus	Compound		
	Approved for medical use	In clinical development	In preclinical evaluation
Pesti (BVDV)	—	Pegylated interferon- α ("off label")	Indole- <i>N</i> -acetamide derivative Benzothiadiazine derivative Phenylalanine derivative Thiophene 2-carboxylic acid derivative Dihydropyranone derivative Tetrahydropyranoindolyl acetic acid derivative HCV-371 <i>N</i> -1-Aza-4-hydroxyquinolone benzothiadiazine A-782759
			VP32947 Compound 1453 BPIP AG110
Corona (SARS)	—	Pegylated interferon- α ("off label")	Calpain inhibitors (III, VI) Niclosamide anilide Phe-Phe dipeptide TG-0205221 Bananin Valinomycin Glycyrrhizin Chloroquine Niclosamide Nelfinavir

(continued on next page)

Table 1. — *Continued*

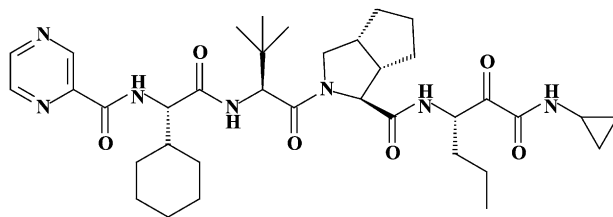
Virus	Compound		
	Approved for medical use	In clinical development	In preclinical evaluation
Orthomyxo (Influenza)	Amantadine	—	RWJ-270201
	Rimantadine		A-192558
	Zanamivir		A-315675
	Oseltamivir		T-705
	Ribavirin ('off label')		Flutimide
Paramyxo (Parainfluenza, Measles, Mumps, RSV, hMPV, ...)	Ribavirin (approved for RSV only)	—	VP-14637
			JNJ-2408068
			BMS-433771
			MBX-300
			A-60444
			Compound D
Arena (Lassa, ...)	Ribavirin ("off label")	—	YM-53403
			BCX 2798 & BCX-2855
Bunya (Crimean–Congo, Rift Valley, ...)	Ribavirin ("off label")	—	—
Rhabdo (Rabies)	—	—	—
Filo (Ebola, Marburg)	—	—	3-Deazaneplancin A

**Pleconaril****Rupintrivir****Pyrrolidinyl pentenoic acid ethyl ester****Mycophenolic acid (MPA) mofetil****Figure 1.** Structural formulae of antiviral compounds.

**Triaryl pyrazoline****BILN 2061
Ciluprevir****Figure 1.** — *Continued.*

potential in the therapy and/or prophylaxis of RSV infections remains to be further evaluated.

Although human parainfluenza viruses are important respiratory tract pathogens, especially in children, they have received little attention from either prophylactic (vaccine) or therapeutic viewpoint. Yet, they contain a unique target, the major surface glycoprotein hemagglutinin-neuraminidase (HN) that serves, at the same time, for cell attachment and virus spread. The HN inhibitors BCX 2798 and BCX 2855 were found to inhibit both functions, and to block infection with parainfluenza viruses both *in vitro* and *in vivo*³ These compounds may limit parainfluenza virus infections in humans. Other compounds which may be further pursued for their activity against parainfluenza



VX-950
Telaprevir

Figure 1. — Continued.

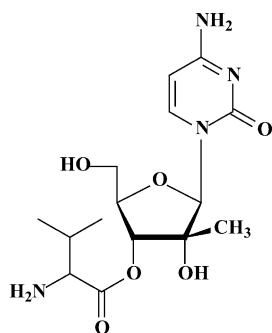
viruses, RSV, as well as influenza viruses, include flavonoids,¹⁸⁷ uncinosides¹⁰⁸ and polyoxotungstates,¹⁴⁸ and diffaeoylquinic acid isolated from the ethnobotanical L-deflexicalyx.¹²¹

VIII. ARENA-, BUNYA-, RHABDO- AND FILOVIRUSES

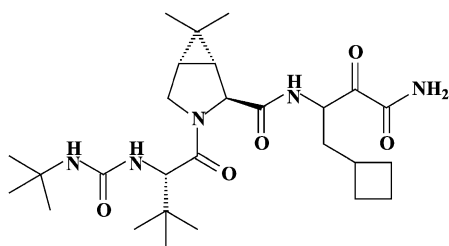
Of the 23 arenaviruses known, five are associated with viral hemorrhagic fever: Lassa, Junin, Machupo, Guanarito and Sabia.¹⁹ Ribavirin has proven to be effective in the post-exposure prophylaxis and therapy of experimental arenavirus infections in animal models, and anecdotal reports suggest that it might also be effective in the treatment of arenavirus infections (i.e. Machupo and Sabia) in humans.¹⁹ The most convincing evidence for the (clinical) efficacy was obtained in the case of Lassa fever, where it was found to reduce the case-fatality rate, irrespective of the time point in the illness when treatment was started¹¹¹

Of equal significance is that in a prospective, double-blind, placebo-controlled clinical trial, intravenous ribavirin therapy was shown to reduce mortality due to Hantaan infections [HFRS (Hemorrhagic Fever with Renal Syndrome)],⁷⁶ which is not surprising as the (–)RNA bunyaviruses, and in particular, hantaviruses, are among the most sensitive RNA viruses to ribavirin *in vitro*.

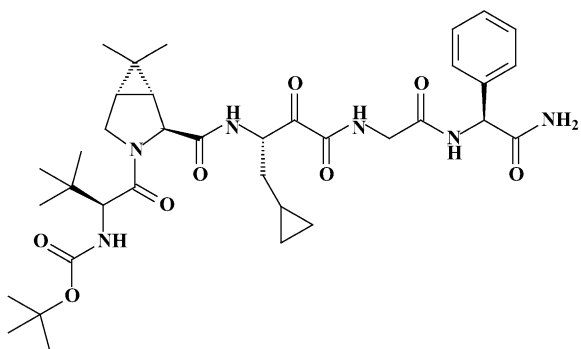
Of the bunyaviruses, one of the most feared (because it is highly infectious, easily transmitted between humans, and associated with a case-fatality rate of approximately 30%) is Crimean–Congo hemorrhagic fever virus.²⁷ Bunyaviruses are sensitive to ribavirin, and this has also been demonstrated in experimental animal models.¹⁴⁹ Also, interferon and interferon inducers have proved effective in the treatment of experimental bunyavirus infections, and, likewise, interferon- α should be considered for the treatment of arenavirus infections, as



NM283
3'-Valine ester of 2'-C-methylcytidine
Valopicitabine

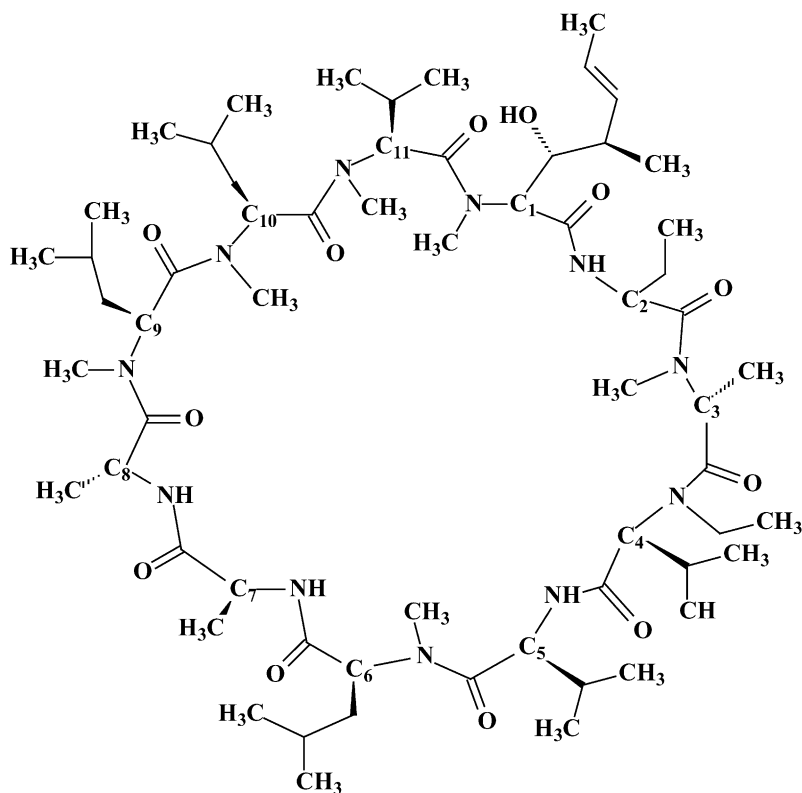


SCH 503034



SCH6 (SCH 446211)

Figure 1. — *Continued.*



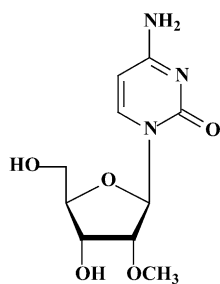
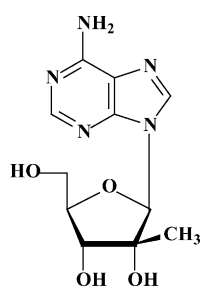
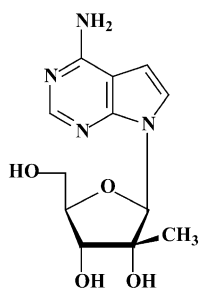
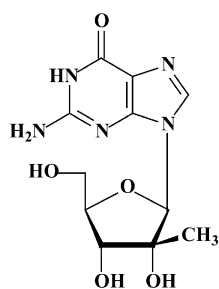
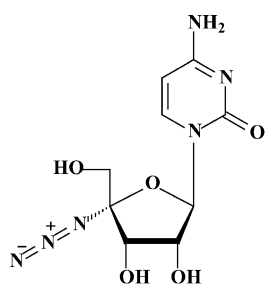
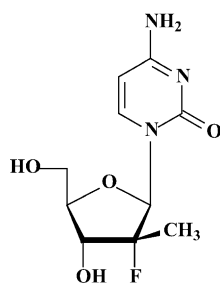
DEBIO-025

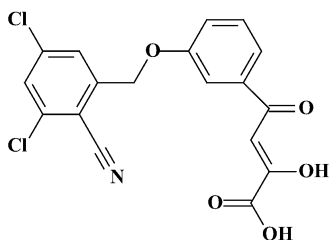
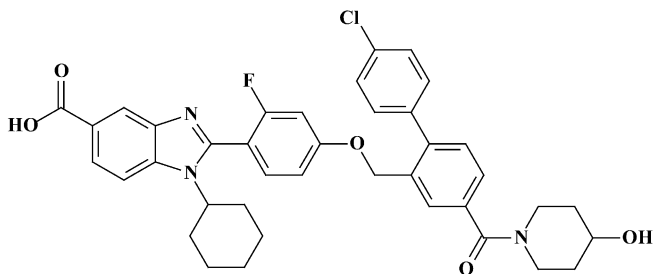
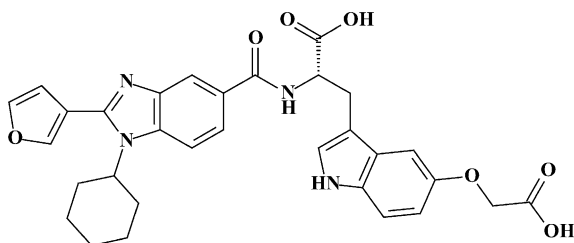
Figure 1. — Continued.

warranted by its efficacy in the therapy of Pichinde virus infection in hamsters.⁵⁶

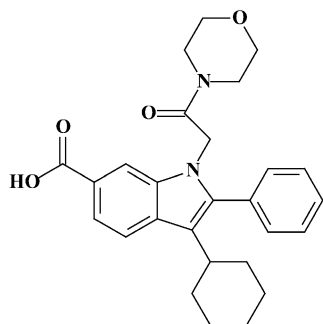
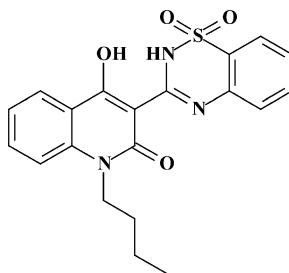
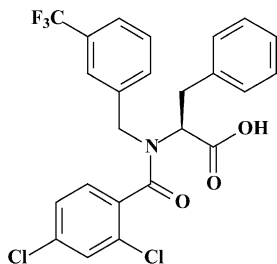
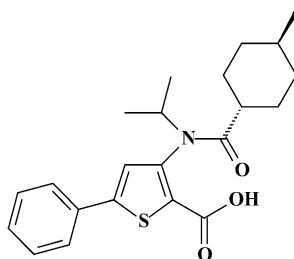
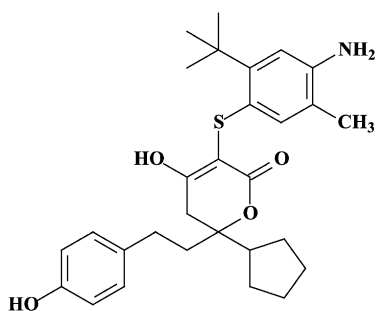
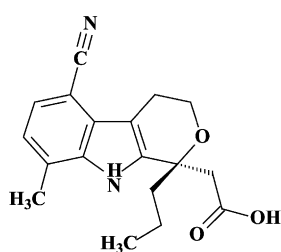
Of the rhabdoviruses, rabies, which is almost invariably fatal if no control measures are taken, can be contained by repeated injections of specific immunoglobulin and/or the inactivated ("killed") rabies vaccine as soon as possible after the infection. For the filovirus infections Ebola and Marburg no vaccine is (yet) available. Specific immunoglobulin or interferon- α may only be of limited value in the treatment of filovirus infections, as indicated by experimental findings in rhesus macaques infected with Ebola (Zaire) virus.⁸⁰

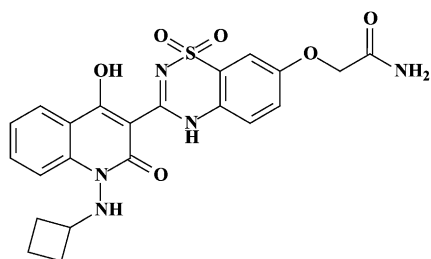
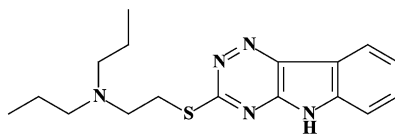
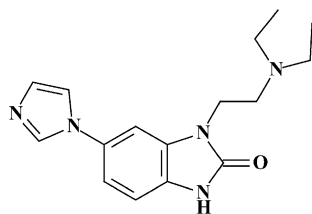
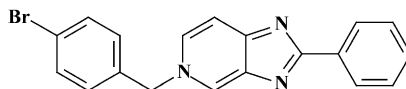
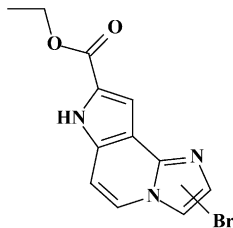
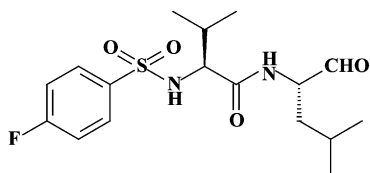
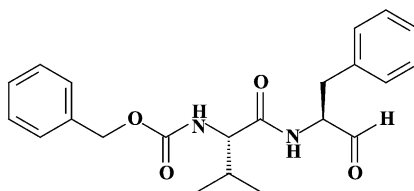
No antiviral drugs that are currently in clinical use, including ribavirin, provide meaningful protection against filoviruses *in vivo*.¹⁰

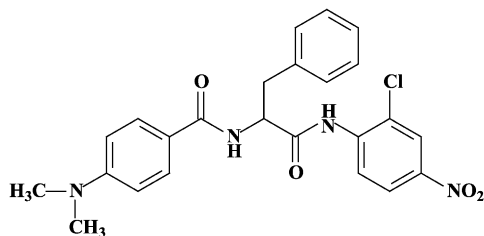
**2'-O-methylcytidine****2'-C-methyladenosine****7-deaza-2'-C-methyladenosine****2'-C-methylguanosine****4'-Azidocytidine
(R1479)****2'-Deoxy-2'-fluoro-2'-C-methylcytidine****Figure 1.** — *Continued.*

**DKA Compound 30****Benzimidazole-based derivative****Benzimidazole 5-carboxamide derivative****Figure 1.** — *Continued.*

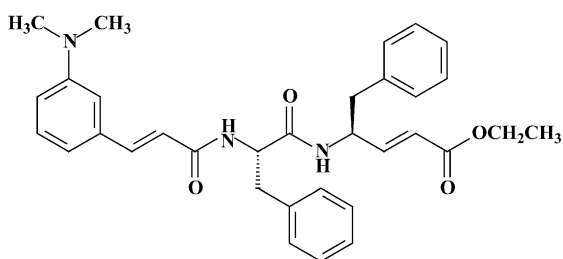
A possible therapeutic strategy may be based on the use of S-adenosylhomocysteine (SAH) hydrolase inhibitors.³¹ SAH hydrolase inhibitors, such as 3-deazaneplanocin A, interfere with S-adenosylmethionine (SAM)-dependent methylation reactions, particularly those involved in the “capping” of viral mRNA. Some viruses, such as the rhabdovirus VSV (vesicular stomatitis virus), heavily rely on mRNA

**Indole-N-acetamide derivative****Benzothiadiazine derivative****Phenylalanine derivative****Thiophene 2-carboxylic acid derivative****Dihydropyranone derivative****Tetrahydropyranoindolyl acetic acid derivative HCV-371****Figure 1.** — *Continued.*

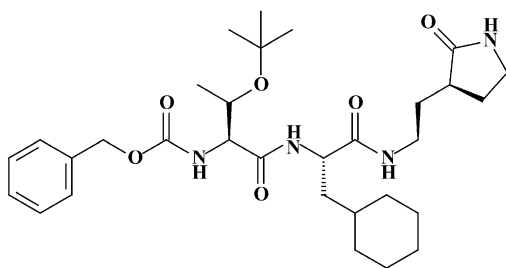
**A-782759****VP32947****Compound 1453****BPIP****AG110****Calpain inhibitor VI****Calpain inhibitor III***Figure 1. — Continued.*



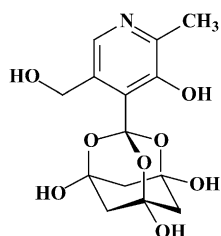
**Niclosamide anilide
(JMF 1507)**



Phe-Phe dipeptide



TG-0205221

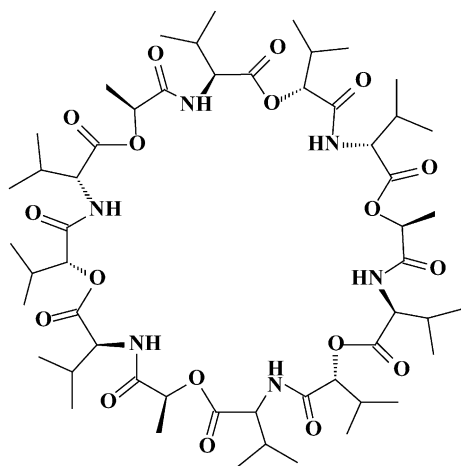


Bananin

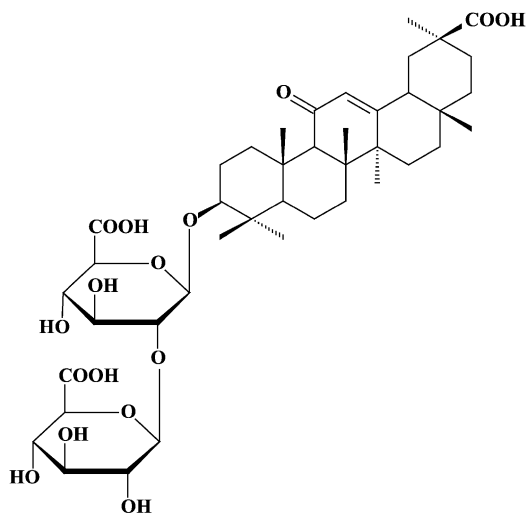
Figure 1. — *Continued.*

capping and are particularly sensitive to inhibition by SAH hydrolase inhibitors, including 3-deazaneplanocin A.³²

As, biochemically, filo- and rhabdoviruses are quite similar in their replication machinery, both requiring 5'-capping of their mRNAs, SAH hydrolase inhibitors such as 3-deazaneplanocin A may logically be expected to be effective in the treatment of Ebola virus infections. In



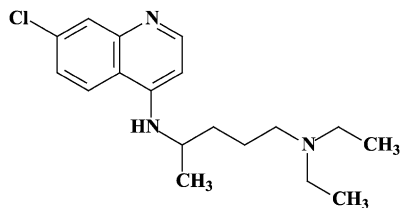
Valinomycin



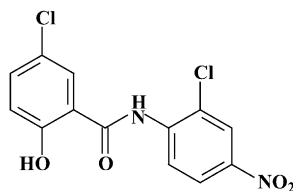
Glycyrrhizin

Figure 1. — Continued.

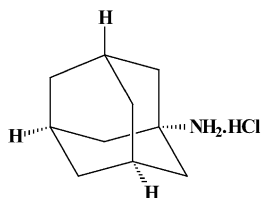
fact, when administered as a single dose of 1 mg/kg, 3-deazaneplanocin A was found to protect mice against a lethal infection with Ebola virus (Zaire strain).^{11,12} This protective effect was accompanied, and proba-



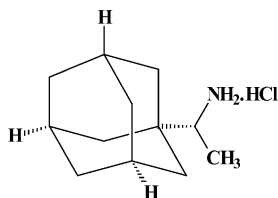
Chloroquine
(Nivaquine)
7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline



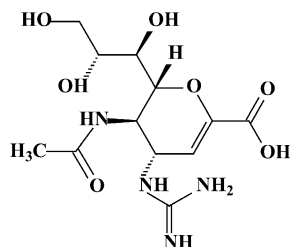
Niclosamide



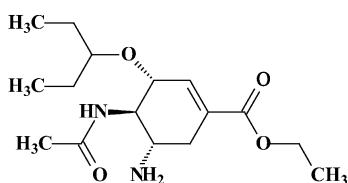
Amantadine
Symmetrel®, Mantadix®



Rimantadine
Flumadine®



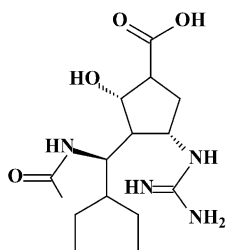
Zanamivir
Relenza®



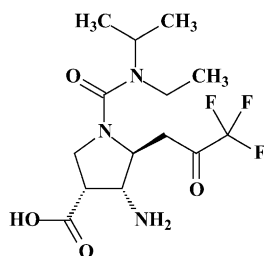
Oseltamivir
Tamiflu®

Figure 1. — Continued.

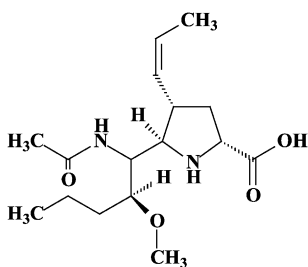
bly mediated, by the production of high concentrations of interferon in the Ebola virus-infected mice.¹² It can be hypothesized that, by blocking the 5'-capping of the nascent (+)RNA viral strands (and, hence, their maturation towards mRNAs), 3-deazaneplanocin A stimulated



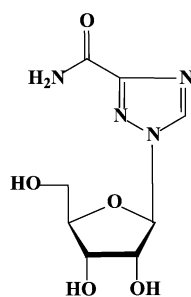
RWJ-270201
Peramivir



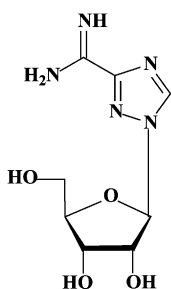
A-192558



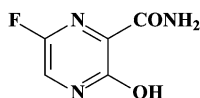
A-315675



Ribavirin



Viramidine

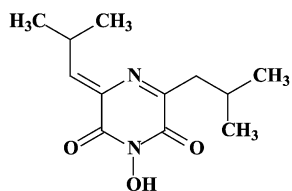
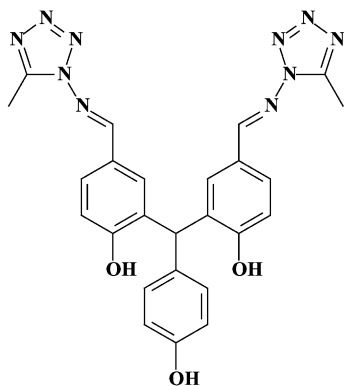
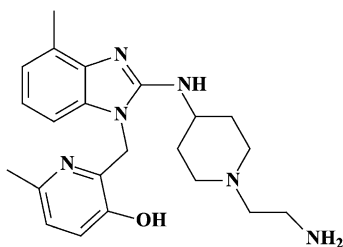
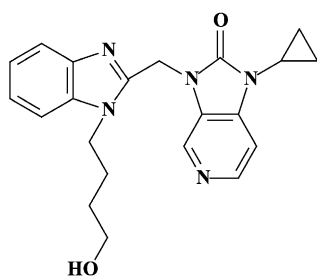
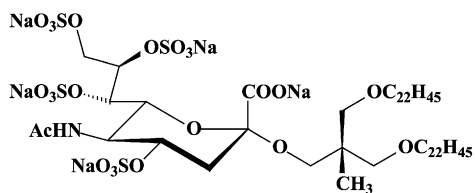
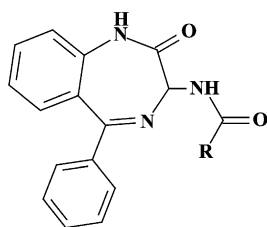


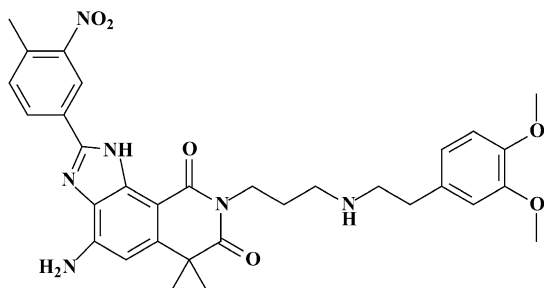
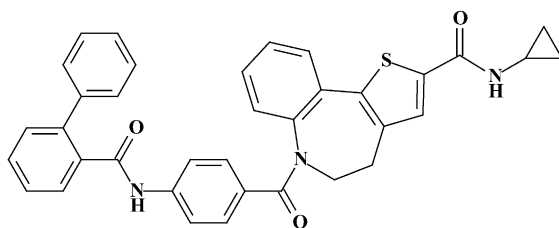
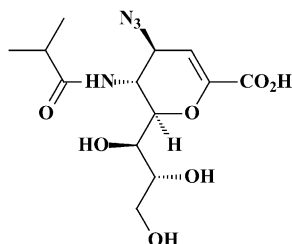
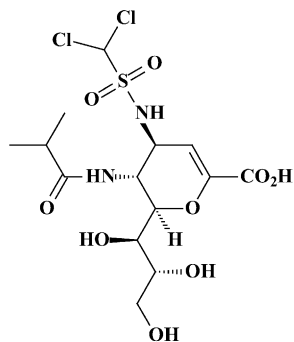
T-705

Figure 1. — Continued.

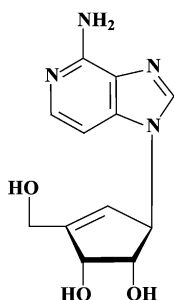
the formation of double-stranded (\pm)RNA complexes, which have since long been known as excellent inducers of interferon.¹⁶

Like SAH hydrolase, IMP (inosine monophosphate) dehydrogenase is another cellular enzyme that may be envisaged as a target for antiviral agents. IMP dehydrogenase is a crucial enzyme involved in the

**Flutimide****VP-14637****JNJ-2408068**
R-170591**BMS-433771****MBX-300****A-60444****Figure 1.** — *Continued.*

**Compound D****YM-53403****BCX-2798****BCX-2855****Figure 1.** — *Continued.*

biosynthesis of GTP, and, although ribavirin may act against distinct viruses by distinct mechanisms (i.e. IMP dehydrogenase inhibition, immunomodulatory effect, RNA capping interference, polymerase in-



3-Deazaneplanocin A

Figure 1. — *Continued.*

hibition, lethal mutagenesis),⁵⁷ the predominant mechanism by which ribavirin exerts its antiviral activity *in vitro* against flaviviruses and paramyxoviruses is mediated by inhibition of IMP dehydrogenase.⁹⁹

Recently, a new class of compounds, phosphorodiamidate morpholino oligomers (PMO), conjugated to arginine-rich cell-penetrating peptides (P-PMO) and designed to base pair with the translation start region of Ebolavirus VP35 positive-sense RNA, were reported to inhibit Ebolavirus replication and to protect mice against a lethal Ebolavirus infection.⁴⁷ Similarly, siRNAs targeting Ebola virus (Zaire) polymerase (L) gene and delivered using SNALPs (stable nucleic acid–lipid particles) were found to completely protect guinea pigs against death when administered shortly after Ebola virus infection.⁵³

IX. REOVIRUSES

Of the reoviruses, rotavirus, which is associated with viral gastrointestinal infections, is by far the most clinically important pathogen. Several attempts have been, and are still being, made to develop an effective vaccine for rotavirus infections. Current treatment for rotavirus diarrhoea is mainly based on the administration of fluids to prevent dehydration. There are no serious attempts to develop an antiviral drug for this disease, although it is noteworthy that the replication of reo- (and rota)viruses is exquisitely sensitive to SAH hydrolase inhibitors such as 3-deazaneplanocin A.³² Also, small interfering RNA (siRNA) corresponding to the VP4 gene has been shown to efficiently inhibit the synthesis of this protein, and to reduce the yield of viral progeny in virus-infected cells.³⁵

X. CONCLUSION

About forty compounds are registered as antiviral drugs, at least half of which are used to treat HIV infections. An even greater number of compounds are under clinical or preclinical development, with again, as many targeting HIV as all the other viruses taken together. This implies that HIV, since its advent, has remained the main stay in antiviral drug development. Antiviral agents can, as guided by the anti-HIV agents as examples, be divided in roughly five categories:

- (i) nucleoside analogues,
- (ii) nucleotide analogues (or acyclic nucleoside phosphonates),
- (iii) non-nucleoside analogues,
- (iv) protease inhibitors, and
- (v) virus–cell fusion inhibitors.

Molecular targets are for (i) and (ii) the viral DNA polymerase (whether DNA-dependent as in the case of herpesviruses, or RNA-dependent as in the case of HIV or HBV); for (iii) RNA-dependent DNA polymerase (reverse transcriptase), associated with HIV, or RNA-dependent RNA polymerase (RNA replicase) associated with HCV; for (iv) the proteases associated with HIV and HCV; and for (v) the fusion process of HIV (and, potentially, other viruses such as the SARS coronavirus and RSV). Antiviral agents may also exert their antiviral effects through an interaction with cellular targets such as IMP dehydrogenase (ribavirin) and SAH hydrolase (3-deazaneplanocin A). The latter enzymes are essential for viral RNA synthesis (through the supply of GTP) and viral mRNA maturation (through 5'-capping), respectively. Finally, interferons (now generally provided in their pegylated form) may be advocated in the therapy of those viral infections (actually, HBV and HCV; prospectively, Coxsackie B, SARS, . . .) that, as yet, cannot be sufficiently curbed by other therapeutic measures.

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METHYLENECYCLOPROPANE ANALOGUES OF NUCLEOSIDES AS ANTI-HERPES AGENTS

Jiri Zemlicka

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ABSTRACT

The current status of methylenecyclopropane analogues of nucleosides has been reviewed including synthetic methodology for the first- and second-generation analogues, antiviral activity with emphasis on β - and γ -herpesviruses, structure-activity relationships and mechanism of action. Fluoromethylenecyclopropanes, nucleotide analogues and phenyl phosphoralaninate (PPA) pronucleotides are also discussed.

I. INTRODUCTION

Methylenecyclopropane analogues of nucleosides are now established antiviral agents with a particular efficacy against β -herpesviruses (cytomegalovirus, CMV; human herpes virus 6, HHV-6) and γ -herpesviruses (Epstein–Barr virus, EBV; human herpes virus 8,

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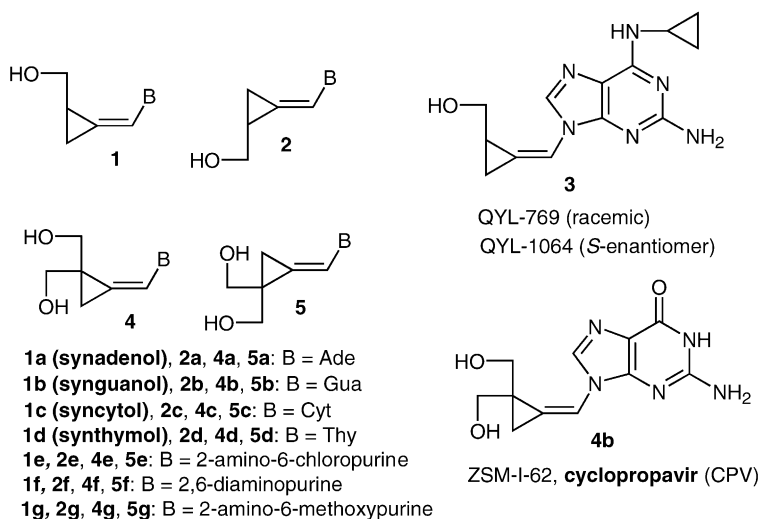


Figure 1.

HHV-8). Less prominent antiviral effects were noted against α -herpesviruses (herpes simplex virus 1 and 2, HSV-1, HSV-2 and varicella zoster virus, VZV) as well as non-herpesviruses (human immunodeficiency virus, HIV-1, hepatitis B virus, HBV).^{78,79} A potency of several methylenecyclopropanes in mice models of HCMV infection is particularly interesting.

In the first generation of analogues, the most potent methylenecyclopropane analogues are the purine *Z*-isomers **1** (Figure 1) of the *S*-configuration although the enantioselectivity depends on the nature of the virus, e.g., effects against HCMV are much more enantioselective than activity against EBV. The pyrimidine analogues and the *E*-isomers **2** are mostly devoid of a significant effect. The most effective analogue in the first generation series **1** comprising a single hydroxymethyl group is (*S*)-(+)-2-amino-6-cyclopropylaminopurine **3**. In the second generation comprising analogues **4** and **5** with two *geminal* hydroxymethyl functions, the spectrum of anti-HCMV activity is more narrow but in this series the most potent methylenecyclopropane described to date, cyclopropavir (CPV, **4b**), was found. In addition, the *E*-series **5** yielded several agents effective against EBV.

We have continued structure–activity relationship studies (SAR) of methylenecyclopropane analogues (Figure 2). The 2-halogenated purines **6a**, **7a**, **6b**, **7b** and isoguanines **8a** and **9a** relate to the

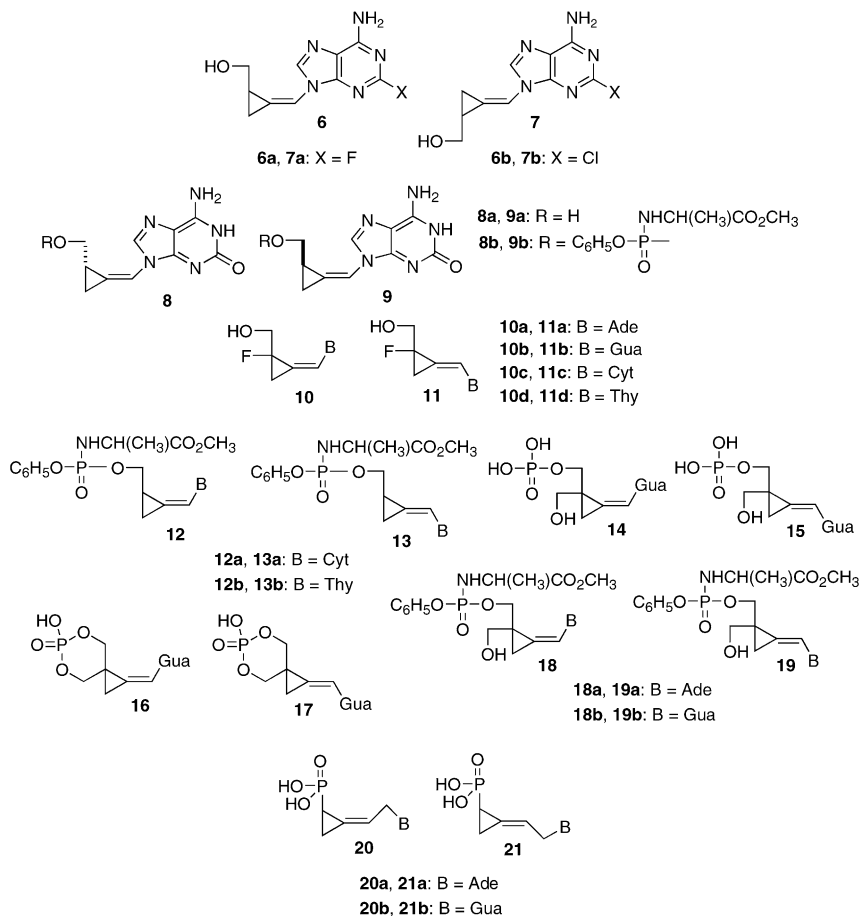


Figure 2.

first-generation analogues **1** and **2**. A new series of fluorinated analogues **10a–10d** and **11a–11d** comprises all major DNA bases. Phosphoramidates **8b**, **9b**, **12a**, **13a**, **12b** and **13b** are then pronucleotides of the respective first-generation methylenecyclopropane analogues. In the second-generation series based on structures **4** and **5**, phosphorylated analogues were studied including cyclopropavir phosphate (**14**), *E*-isomer **15**, cyclic phosphates **16**, **17** and pronucleotides **18a**, **19a**, **18b**, **19b**. Newly designed methylenecyclopropane phosphonates **20a**, **20b**, **21a** and **21b** were also in the focus of our investigation.

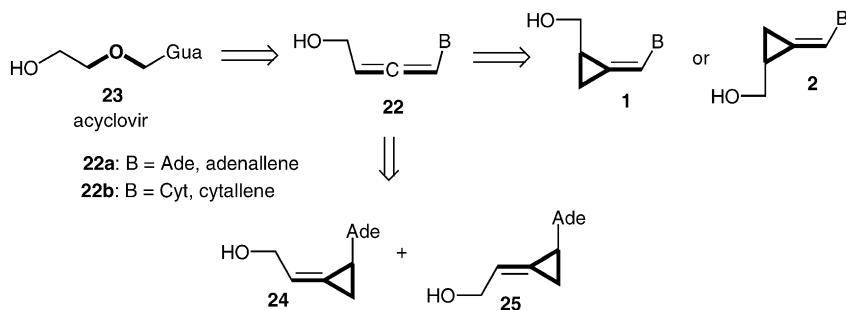


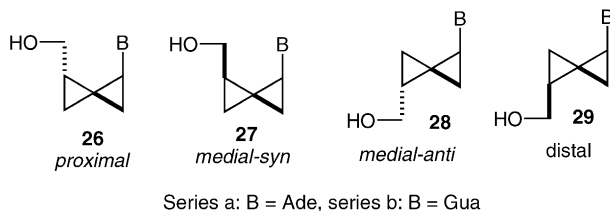
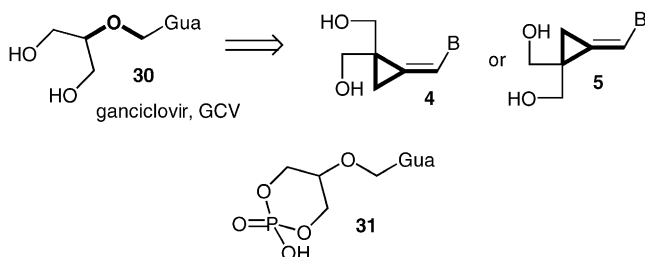
Figure 3.

Because the subject of methylenecyclopropane analogues of nucleosides has been recently reviewed,^{78,79} this account will put emphasis on general conclusions and new findings.

II. DESIGN

Our early efforts in the design and synthesis of new nucleoside analogues focused on allenic derivatives **22** (Figure 3).^{75,76} The computer-assisted overlap of adenallene (**22a**) indicated a similarity with the 3'-endo form of adenosine. The distances between the adenine and hydroxymethyl functions were also close.⁴⁵ We have also recognized that the allenic function C=C=C is isosteric and isoelectronic with C–O–C moiety found in antiherpetic agent acyclovir (**23**). Adenallene (**22a**) and cytallene (**22b**) exhibit a potent anti-HIV activity^{23,75} and the latter analogue is a strong anti-HBV agent.⁸¹ For reviews of allenic analogues see Refs. [24,26,75,76].

Encouraged by these results, we attempted to extend this analogy. It is well known that cyclopropane has bent bonds⁵⁹ which are intermediate between the σ and π bonds. Cyclopropanes may then be regarded as analogues of unsaturated compounds. Replacement of the *distal* double bond in allenes **22** with a cyclopropane ring leads to *Z*- and *E*-isomers **1** and **2**. The *Z*- and *E*-analogues **24** and **25** are derived in a similar fashion by replacing the *proximal* double bond of allenes **7**. When both double bonds are replaced, four isomeric spiropentanes **26**, **27**, **28** and **29** are obtained (Figure 4). A potent anti-EBV effect was noted with adenine *proximal* and guanine *distal* spiropentanes **26a** and **29b**,¹⁸ whereas adenine analogues **24** and **25**⁵⁴ lack a significant antiviral potency. The latter results indicated that orientation

**Figure 4.****Figure 5.**

of the methylenecyclopropane “linker” relative to the hydroxymethyl group and heterocyclic base is of decisive importance for antiviral activity. The bond length of the alkene C=C bond in *R*-(*-*)-synadenol /(*R*)-**1a**/ obtained by X-ray diffraction (1.319 Å)⁴⁹ is 0.018 Å shorter than in methylenecyclopropane itself (1.337 Å).⁵⁶

As indicated at the outset, the allenic function is isoelectronic and isosteric with the C–O–C found in acyclovir (**23**, Figure 3). By contrast, methylenecyclopropane function of analogues **1** and **2** is not isoelectronic with the respective allenic or C–O–C moiety, but they can be regarded as bioisosteres.³⁶ In such a way, the guanine analogues of the first generation series **1** and **2** are bioisosteric with acyclovir (**23**), whereas the second generation compounds **4** and **5** are bioisosteres of ganciclovir (**30**, Figure 5).

As indicated in the Introduction, both first- and second-generation methylenecyclopropane analogues provided a number of effective antiviral agents. Compounds **6a**, **7a**, **6b**, **7b**, **8a**, **9a** and fluorinated analogues **10a–10d**, **11a–11d** can then be considered as an extension of the SAR studies in the first-generation series **1** and **2**. In addition, introduction of fluorine into biologically active compounds led to analogues with new activities⁷² including antiviral nucleosides.⁴⁴

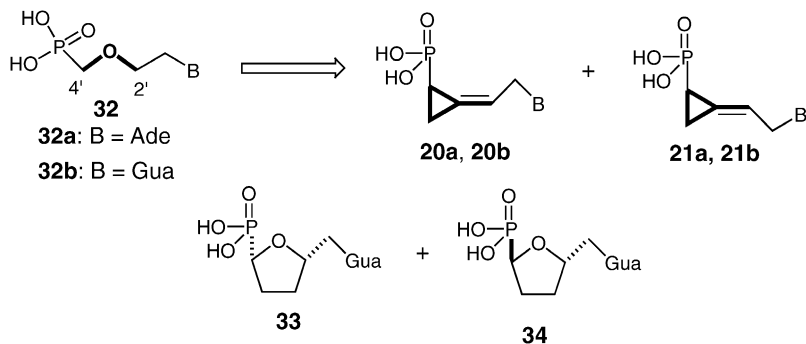


Figure 6.

Pronucleotides **8b**, **9b**, **12a**, **13a**, **12b** and **13b** also relate to the first-generation methylenecyclopropanes. In the second-generation series **4** and **5**, nucleotide analogues of cyclopropavir (**4b**), included phosphates **14** and **15**. Compound **14** can be considered a prodrug of cyclopropavir (**4b**) with an increased solubility. The rationale for the cyclic phosphates **16** and **17** is based on a similar derivative of ganciclovir **31** (Figure 5),^{15,17,43,63} which exhibited potent antiviral effects. The intramolecular distance between the guanine N-9 and phosphorus atom of **16** (5.944 Å)⁷³ is close to that found in **31** (5.893 Å).^{5,6} Because several phenyl phosphoralaninates of methylenecyclopropane purine analogues **1** and **2** exhibit antiviral effects more potent than the parent analogues,^{52,64,77} pronucleotides **8b**, **9b**, their pyrimidine counterparts **12a**, **12b**, **13a** and **13b** as well as second-generation phosphoramidates **18a**, **18b**, **19a**, **19b** were also included in our studies.

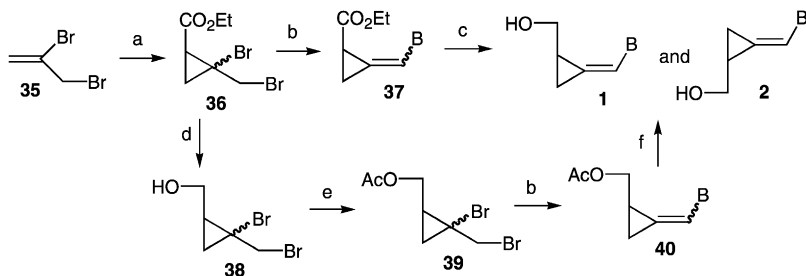
Design of phosphonates **20a**, **20b**, **21a** and **21b** is an extension of the methylenecyclopropane rationale (Figure 6). Acyclic nucleoside phosphonates such as **32a** are potent antiviral agents.²⁵ In analogy to acyclovir (**23**, Figure 3), the replacement of C–O–C group in **32a** and **32b** with methylenecyclopropane moiety leads to phosphonates **20a**, **20b**, **21a** and **21b**. This would limit rotatable bonds from five in acyclic phosphonates **32** to three in methylenecyclopropanes **20** or **21**. Interestingly, another pair of tetrahydrofuran phosphonates **33** and **34** with only three rotatable bonds derived by connecting two carbon units with the 2' and 4' positions of **32b** exhibit potent anti-HCMV activity.^{3,40,41} The *trans* analogue **34** is also an antitumor agent.^{7,34}

III. SYNTHESIS

A. First-generation methylenecyclopropane analogues

1. Racemic analogues

An approach permitting introduction of the methylenecyclopropane moiety into any nucleic acid base or suitable precursor was considered as the most preferable. Alkylation–elimination method described in Figure 7 fulfills such a requirement. It was first applied for synthesis of racemic methylenecyclopropanes. Addition of carbene derived from ethyl diazoacetate to 2,3-dibromopropene (**35**) gave dibromocyclopropane carboxylate **36**.⁵⁰ A one-pot alkylation–elimination of adenine with **36** gave the *Z*- and *E*-isomeric methylenecyclopropanes **37**. Reduction then afforded synadenol (**1a**) and the corresponding *E*-isomer **2a**. Alternately, dibromocyclopropane **36** was reduced to alcohol **38** which was then acetylated to give reagent **39**.⁴⁸ Alkylation–elimination with adenine gave intermediate **40** and, after deacetylation, synadenol (**1a**) and the *E*-isomer (**2a**). The isomers **1a** and **2a** were separated by chromatography on silica gel after derivatization using dimethylformamide dimethyl acetal. To avoid derivatization, compound **38** was protected with methoxymethyl (MOM) group and the resultant reagent **41** was used for alkylation–elimination (Figure 8). Intermediates **42** and **43** were separated by chromatography to furnish, after deprotection, synadenol (**1a**) and the *E*-isomer **2a**.⁴⁸ This is currently the best procedure for preparation of synadenol (**1a**).^{69,71} Alkylation–elimination procedure was also successful with other purine derivatives such as 2-amino-6-chloropurine⁵⁰ and 2,6-diaminopurine.⁴⁶ In these instances,



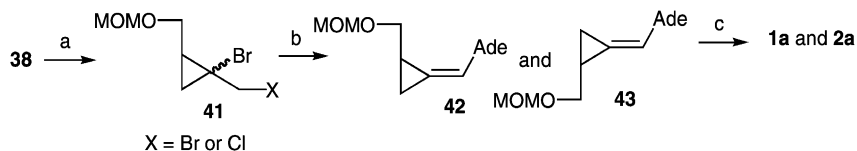
For series a - d see Figure 1, series e: B = 2-amino-6-chloropurine, series f: B = 2,6-diaminopurine

a. $\text{N}_2\text{CHCO}_2\text{Et}$, $\text{Rh}_2(\text{OAc})_2$ (cat.), CH_2Cl_2 .
b. B-H, K_2CO_3 , DMF, Δ .

c. DIBALH, THF.
d. DIBALH, hexane.

e. Ac_2O , pyridine.
f. B, K_2CO_3 , $\text{MeOH}/\text{H}_2\text{O}$.

Figure 7.



a. $\text{CH}_3\text{OCH}_2\text{Cl}$ (MOMCl), $\text{Et}(\text{-i-Pr})_2\text{N}$, CH_2Cl_2 . b. Adenine, K_2CO_3 , DMF, Δ . c. HCl, MeOH.

Figure 8.

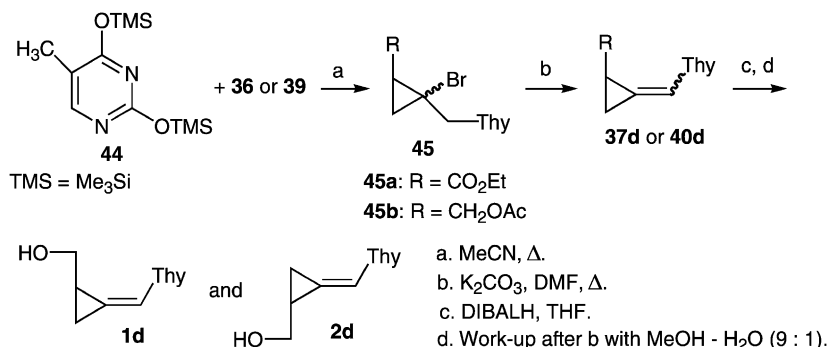
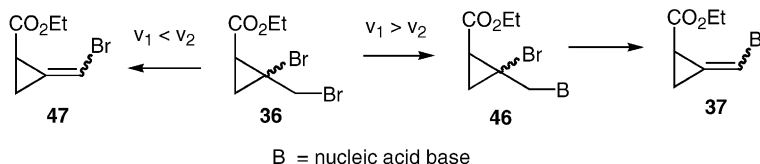
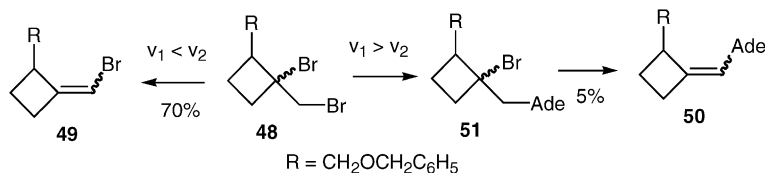


Figure 9.

analogues **1e** and **1f** were separated from the *E*-isomers **2e** and **2f** without derivatization.

In the pyrimidine series, alkylation–elimination of N^4 -acetylcytidine with reagents **36** or **39** proceeded uneventfully (the N^4 -acetyl group was hydrolyzed during the work-up of the reaction mixture,^{1,51} but derivatization (N^4 -benzoylation) was employed in order to separate syncytol (**1c**) from the *E*-isomer (**2c**). By contrast, thymine and reagent **36** gave a complex mixture of products and, therefore, an alternate approach which separated alkylation and elimination step was adopted (Figure 9). A prolonged reflux of silylated thymine **44** with dibromoester **36**⁵¹ or acetate **39**¹ afforded the bromo intermediates **45a** or **45b**. The elimination was accomplished as described in Figure 7 to give the *Z*, *E*-isomers **37d** or **40d**. Reduction (**37d**) or deacetylation (**40d**) afforded the *Z*- and *E*-isomers **1d** and **2d** which were separated by chromatography.

It is important to note that a success of alkylation–elimination procedure described above (Figure 7) depends on the relative rates of alkylation (v_1) and elimination (v_2) of the reagent, e.g., dibromocyclopropane carboxylate **36**. If the v_1 is greater than v_2 , an alkylation

**Figure 10.****Figure 11.**

product **46** forms first and elimination ensues in the second step to give methylenecyclopropane **37** (Figure 10) and the formation of bromoalkene **47** is suppressed. Alkylated intermediates such as **46** (B = Ade) were isolated,⁵⁰ see also compounds **45a** or **45b** (Figure 9) resultant from alkylations with reagents **36** or **39** in the absence of base. In case that $v_1 < v_2$, alkylation reagent **36** is predominantly transformed to an unreactive bromoalkene **47**. In the series of methylenecyclopropane analogues, the type of substituent in the cyclopropane portion did not play a decisive role in the direction of elimination. Thus, compounds **37** and **40** are always the major products.^{48,50,51}

The opposite situation when $v_1 < v_2$ is exemplified by dibromocyclobutane derivative **48**¹⁹ where alkylation–elimination led to bromoalkene **49** as the major product (Figure 11) and formation of **50** by elimination of HBr from **51** proceeded only to a negligible extent.

Alkylation–elimination of nucleic acid bases with dibromocyclopropanes **36** or **39** leads to a mixture of *Z*- and *E*-isomers of the resultant methylenecyclopropanes in the ratio of 2 : 1 (**37**) or 1 : 1 (**40**). It may then seem that method employing carboxylate **36** giving more of the *Z*-isomer **37** is of advantage over the procedure using acetoxycyclopropane **39**. However, this is offset by a more difficult work-up of the reaction mixtures after the reduction of methylenecyclopropanes **37** with DIBALH (Figure 7).

Reactive chlorine atom of 2-amino-6-chloro derivative **1e** is readily substituted by nucleophiles using general procedures of nucleoside

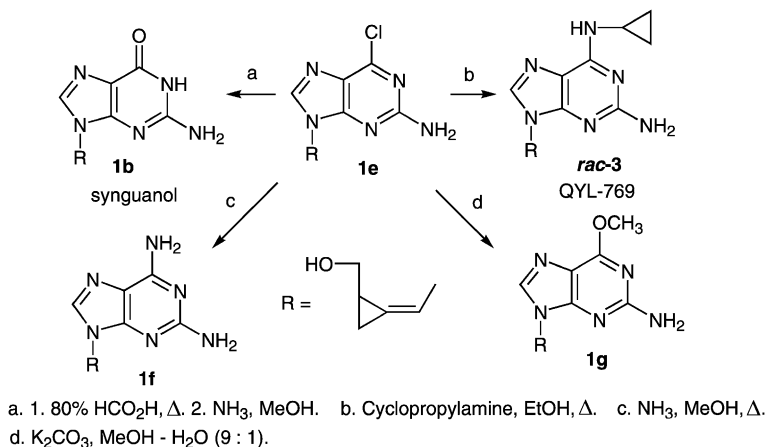


Figure 12.

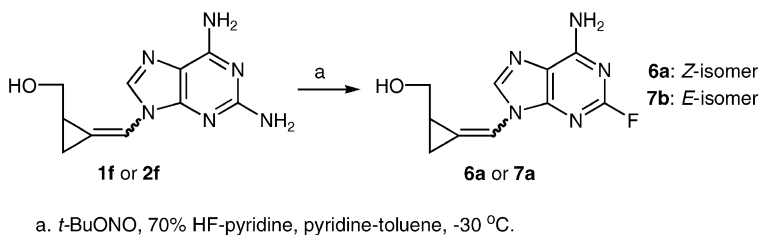


Figure 13.

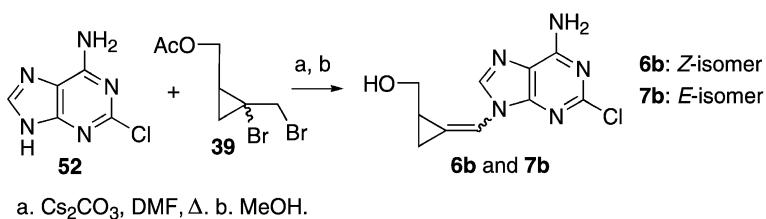


Figure 14.

chemistry.^{50–52} This is shown on examples of antiviral methylenecyclopropanes **1b**, **1f**, **1g** and **rac-3** in Figure 12.

Methylenecyclopropane analogues comprising other substituents other than amino group in the purine 2 position were also a part of structure-activity relationships studies. Thus, 2-fluorosynadenol (**6a**)

and the *E*-isomer **7a** were obtained by diazotization/fluorination⁵⁵ of 2,6-diaminopurine analogues **1f** and **2f** (Figure 13).⁴⁶ Because a similar chlorination procedure failed, the chloro derivatives **6b** and **7b** were prepared (Figure 14) by alkylation–elimination of 2-chloro-6-aminopurine (**52**) with reagent **39**.

2. Enantiomeric analogues

Methylenecyclopropane analogues of nucleosides **1** and **2** comprise a center of asymmetry at the carbon carrying the hydroxymethyl function. Initially, the antiviral activity of methylenecyclopropane analogues was investigated with racemic compounds. In the process of exploring the structure-activity relationships it was imperative to study enantiomeric forms of these analogues. Racemic synadenol (**1a**) was resolved by adenosine deaminase-catalyzed deamination,⁴⁹ which selectively deaminated the *S*-(+)-enantiomer to give inosine analogue **53** leaving the *R*-(-)-enantiomer (**R**)-**1a** intact (Figure 15). The latter enantiomer was used to determine the absolute configuration. The deamination product **53** was then converted to *S*-(+)-synadenol /(**S**)-**1a**/ by acetylation, chlorination and ammonolysis.

Obviously, this method is applicable only to analogues which are substrates for adenosine deaminase. Therefore, a more general approach was needed. The initial procedure (Figure 16) started from (*S*)-methylenecyclopropane carboxylic acid (**54**).³² The latter was esterified to give ester **55** which was then transformed to the *S*-configured 2-amino-6-chloropurine enantiomers (**S**)-**1e** and (**S**)-**2e** by a procedure described for racemic compounds⁵³ (Figure 7). The enantiomers (**R**)-**1e** and (**R**)-**2e** were obtained from (*R*)-methylenecyclopropane carboxylic acid (**R**)-**54**,³² along similar lines.

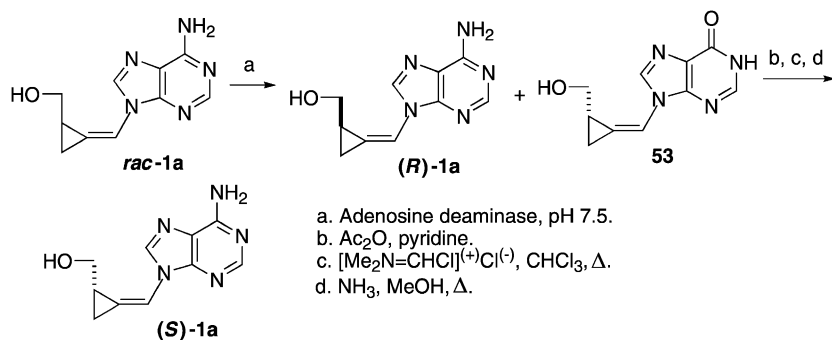
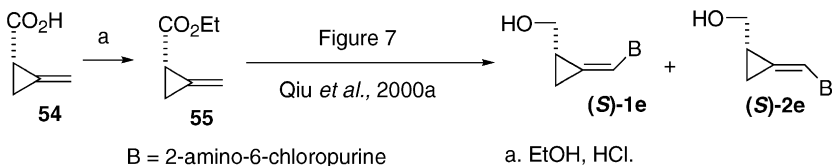
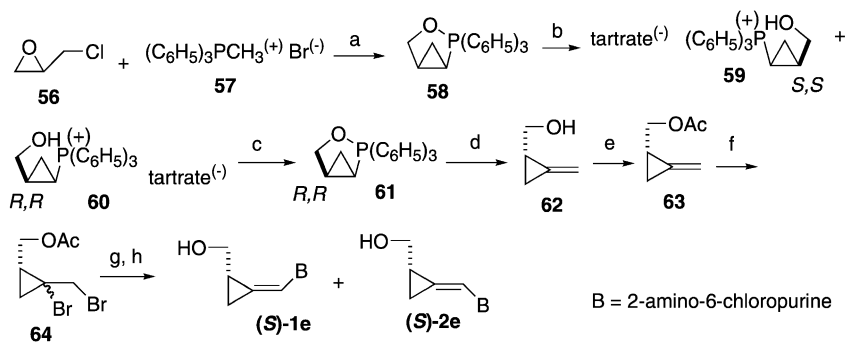


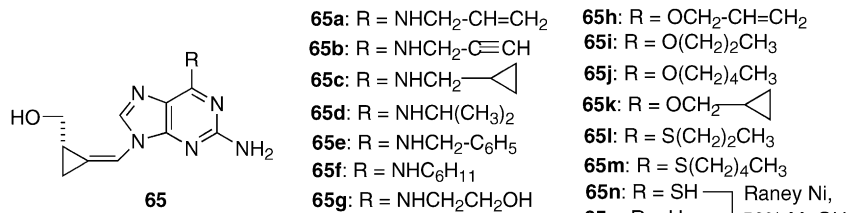
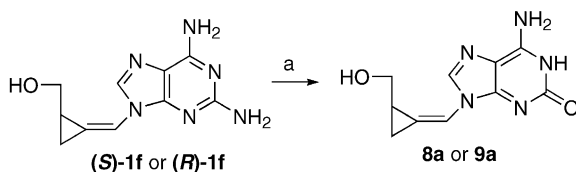
Figure 15.

**Figure 16.**

a. *t*-BuOK, toluene, Δ . b. 1. L-(+)-tartaric acid, Me₂CO. 2. Recrystallization. c. 2M NaOH, CH₂Cl₂. d. (CH₂O)_n, sulfolane, Δ . e. Ac₂O, pyridine. f. Pyridine.HBr₃, CH₂Cl₂. g. 2-Amino-6-chloropurine, Cs₂CO₃, DMF, Δ . h. 1. K₂CO₃ (1 eq.), CH₂Cl₂-MeOH-H₂O (5 : 2 : 1).

Figure 17.

Enantiomeric *Z*-isomers (**(S)-1b** and **(S)-2b**) were the key starting materials for preparation of a number of 2-amino-6-substituted purine analogues^{10,53} used for studies of enantioselectivity and structure-activity relationships. Nevertheless, the resolution method of **Figure 16** suffered from a limited suitability for a large-scale synthesis because a multiple chromatography⁵³ was necessary to obtain **(S)-54** and **(R)-54** in sufficient optical purity (95%). This drawback was removed by an approach⁹ starting from racemic epichlorohydrin (**56**, **Figure 17**). Reaction with Wittig reagent **57** gave oxaphospholane **58** which was resolved by crystallization of the tartrate salt³³ to give *S*, *S*- and *R*, *R*-tartrates **59** and **60**. The *R*, *R*-diastereoisomer **60** was converted to free oxaphospholane **61**. The Wittig reaction with paraformaldehyde gave (*S*)-(+)-methylenecyclopropane carbinol (**62**). Acetylation (**63**) and addition of bromine provided the reagent **64** for alkylation–elimination to give, after deacetylation, the *Z*- and *E*-enantiomers **(S)-1e** and **(S)-2e**. The *R*-enantiomers **(R)-1e** and **(R)-2e** were then obtained from the *S*, *S*-diastereoisomeric salt **59** via **(R)-(-)-63** using a similar procedure.

**Figure 18.**

a. *t*-BuONO or NaNO₂, 80% AcOH.

Figure 19.

The previously assigned⁴² configurations *S* to (–)-**62** and *R* to (+)-**62** that were also adopted by Le Corre et al.³³ are erroneous as shown by Chen and Zemlicka.⁹

Analogues **65a–65n** obtained from intermediate (*S*)-**1e** by reaction with appropriate nucleophiles (see also Figure 12) that were used for investigating the SAR¹⁰ are shown in Figure 18. 6-Deoxypurine analogue **65o** was prepared by desulfurization of 6-thiosynguanol (**65n**).⁷⁰ Isoguanine compounds **8a** and **9a** were obtained from the corresponding 2,6-diaminopurine enantiomers (*S*)-**1f** and (*R*)-**1f** by hydrolytic diazotization with *tert*-butyl or sodium nitrite (Figure 19).⁴⁶

B. Second-generation methylenecyclopropane analogues

Unlike the first generation analogues **1** and **2**, compounds **4** and **5** are achiral which simplified the synthetic approach. Alkylation–elimination method (Figure 7) proved also useful for synthesis of this series of analogues.⁸² The procedure commenced with radical bromination of isopropylidenemalonate **66**⁶⁵ with N-bromosuccinimide (NBS) and dibenzoyl peroxide to give bromoisopropylidene malonate **67** (Figure 20). Reaction with potassium *tert*-butoxide in *tert*-butyl alcohol gave a 1 : 1 mixture of methylenecyclopropane dicarboxylate **68** and isopropylidenemalonate **66** formed by a transfer of positive bromine

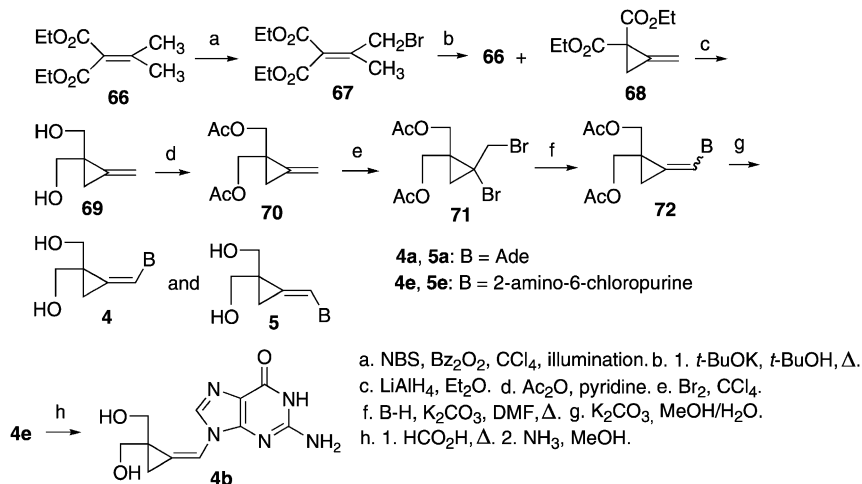


Figure 20.

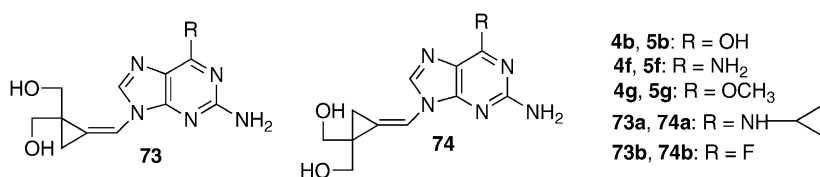


Figure 21.

of **67** to reagent or solvent. Both products were separated by chromatography. Diester **68** was reduced with LiAlH₄ to the corresponding diol **69** which was in turn acetylated to give diacetate **70**. Addition of bromine afforded the reagent **71**. Alkylation–elimination of purine base with **71** as shown for adenine and 2-amino-6-chloropurine followed the procedures elaborated previously for the first-generation analogues (Figure 7) to afford a mixture of the *Z*/*E*-isomeric acetates **72**. Deprotection and chromatographic separation furnished the *Z*- and *E*-isomers **4a**, **5a** or **4e**, **5e**. Compounds **4e** and **5e** were used for a synthesis of 6-substituted purine analogues⁸² including cyclopropavir (**4b**), the *E*-isomer **5b** and compounds **4f**, **5f**, **4g**, **5g**, **73a**, **74a**, **73b**, **74b** (Figure 21). Pyrimidine analogues **4c**, **4d**, **5c** and **5d** were obtained using reagent **71** along the lines described in Figures 7 and 9.

The necessity of separation of intermediates **66** and **68** early in the synthesis is a drawback of this approach. Therefore, a new method

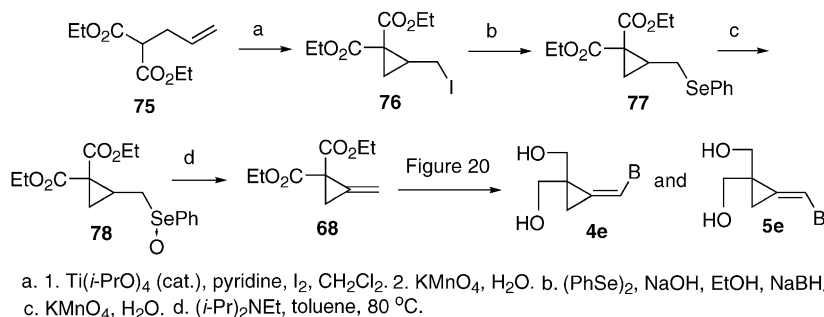


Figure 22.

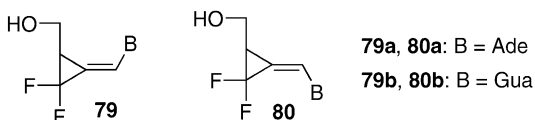


Figure 23.

was elaborated to remove this obstacle (Figure 22).⁷³ Allyl malonate **75** was transformed to iodomethyl diester **76**.²⁹ Reaction with sodium phenylselenenide generated *in situ* gave phenylselenenyl derivative **77**. Oxidation to selenoxide **78** followed by alkene-forming elimination furnished methylenecyclopropane dicarboxylate **68**, which was transformed to *Z*- and *E*-isomers **4e** and **5e** as described in Figure 20.

C. Fluorinated methylenecyclopropane analogues

The first members of this series, *geminal* difluoro analogues **79a**, **79b**, **80a** and **80b** were prepared by alkylation–elimination method⁶⁸ (Figure 23). This approach was not successful for synthesis of monofluoro compounds **10a–10d** and **11a–11d**. Thus, methylenecyclopropane carboxylate **rac-55**¹⁸ was fluorinated with *N*-fluorobenzenesulfonimide (NFSI) using lithium dimethylamide (LDA) in the presence of a large excess of LiCl to give the corresponding fluoroester **81** (Figure 24).⁸⁴ Addition of bromine (**82**), reduction (**83**) and acetylation gave the reagent **84** which was used for alkylation–elimination of adenine. Under a variety of conditions, the yields of product **85** did not exceed 10%.

In a successful approach, (*Z,E*)-methylenecyclopropane carboxylic esters **37a**, **37c** and **37d** (Figures 7 and 9)^{50,51} served as convenient

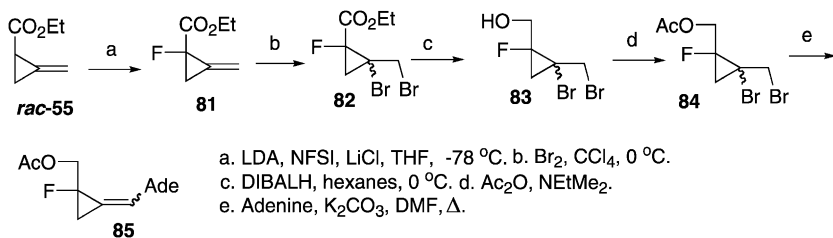


Figure 24.

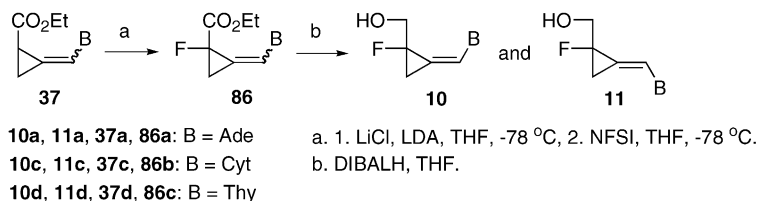


Figure 25.

starting materials.⁸³ Direct fluorination of **37a** as described in Figure 24 for fluoroester **81** gave the (*Z,E*)-isomers **86a** (Figure 25). Reduction with LiBH₄ afforded the *Z*- and *E*-isomers **10a** and **11a** which were separated by chromatography. The cytosine and thymine analogues **10c**, **11c**, **10d**, **11d** were obtained in a similar fashion from esters **37c** and **37d** via intermediates **86b** and **86c**. The (*Z,E*)-2-amino-6-chloropurine esters **37e** were poorly suitable for fluorination. Therefore, they were converted to 2-amino-6-methoxypurine intermediates **87** with K₂CO₃ in MeOH with simultaneous reesterification to the methyl ester. The latter compound was fluorinated without difficulty to give the (*Z,E*)-fluoroesters **88** (Figure 26). Reduction of **88** with DIBALH gave the (*Z*)- and (*E*)-2-amino-6-methoxypurine derivatives **89** and **90** that were separated by chromatography. *O*-Demethylation with KI and Me₃SiCl in acetonitrile³⁹ gave the target guanine analogues **10b** and **11b**.

Although the method described above (Figures 25 and 26) afforded the requisite fluorinated analogues **10a–10d** and **11a–11d**, it required preparation of the individual starting carboxylic esters **37a**, **37c**, **37d** and **37e** (see Figure 7). Also, as already mentioned, a direct and selective fluorination of 2-amino-6-chloropurine ester **37e** could not be achieved. The presence of reactive 2-amino-6-chloropurine base in fluorinated methylenecyclopropanes would be of significant advantage

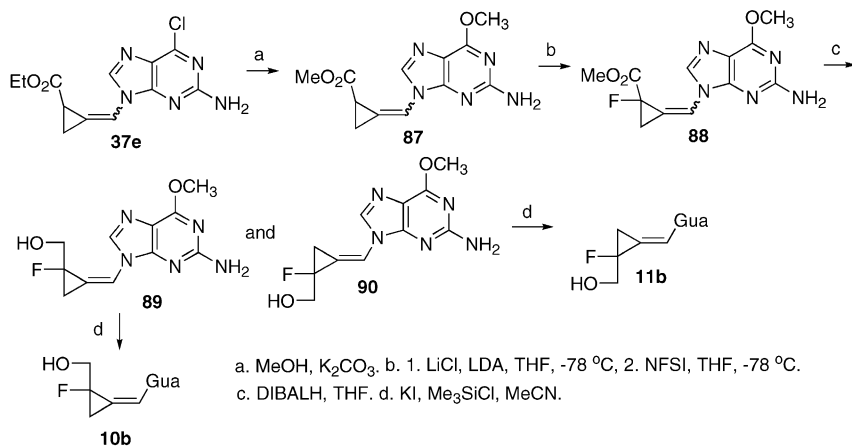
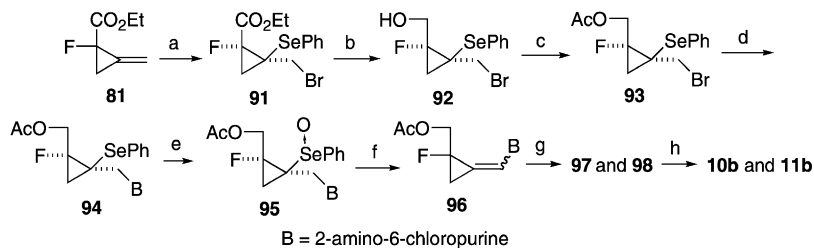


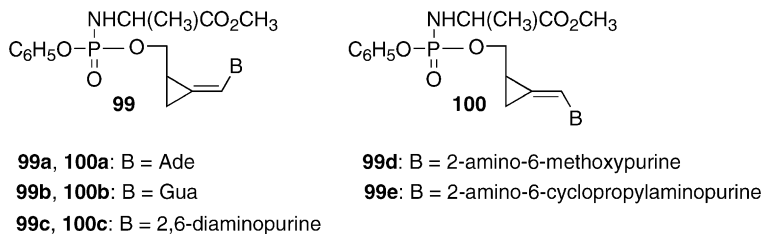
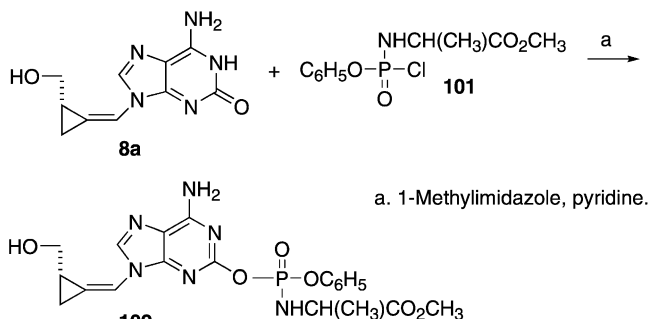
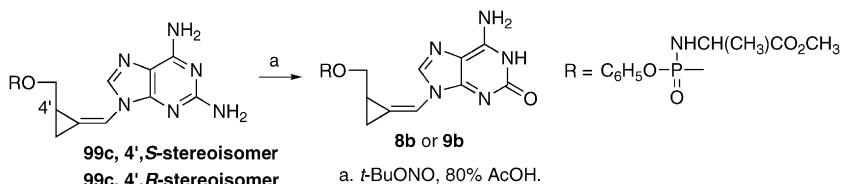
Figure 26.



a. NBS, Ph₂Se₂, CH₂Cl₂. b. DIBALH, THF. c. Ac₂O, pyridine. d. 2-Amino-6-chloropurine, K₂CO₃, DMF. e. H₂O₂, THF. f. THF - DMF (10 : 1), Δ. g. K₂CO₃, MeOH-H₂O (9:1). h. 1. HCO₂H, Δ. 2. NH₄OH.

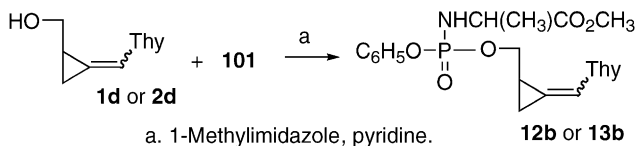
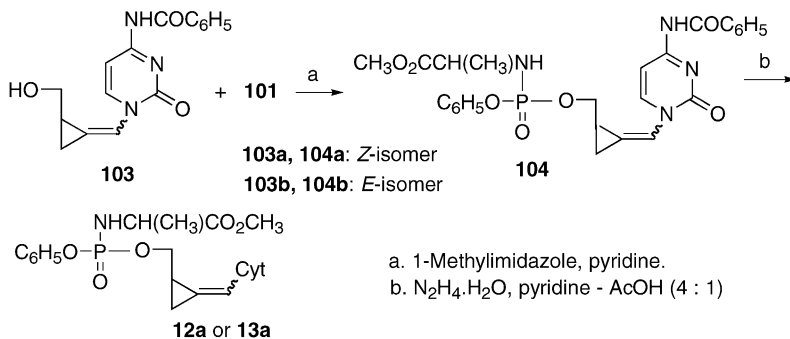
Figure 27.

for the synthesis of 2-amino-6-substituted analogues (see Figures 12 and 18). Avoiding a strong base (LDA, see Figures 25 and 26) as well as separation of alkylation and elimination steps resolved these problems.⁸⁴ Thus, addition of phenylselenenyl bromide generated *in situ* to fluorinated ester **81** (Figure 24) afforded phenylselenenyl bromide **91** of the *E* configuration (Figure 27). Reduction (**92**) followed by acetylation gave alkylating reagent **93** which was used for alkylation of 2-amino-6-chloropurine to furnish intermediate **94**. Oxidation (**95**) followed by elimination of phenylselenoxide function gave the (*Z*, *E*)-methylenecyclopropanes **96**. Deacetylation led to the *Z*- and *E*-isomers **97** and **98** which were separated by chromatography. Hydrolysis with formic acid gave guanine analogues **10b** and **11b**.

**Figure 28.****Figure 29.****Figure 30.**

D. Pronucleotides and nucleotides of methylenecyclopropane analogues

Synthesis of isoguanine phosphoralaninate pronucleotides **8b**, **9b** and pyrimidine derivatives **12a**, **12b** and **13a**, **13b** departed significantly from the procedures elaborated for the respective purine analogues of the first-generation methylenecyclopropanes **99a–99e** and **100a–100c** (Figure 28).⁵² Thus, reaction of isoguanine methylenecyclopropane **8a** with reagent **101** gave only *O*-phosphorylated product **102** of the lactim tautomeric form but the hydroxy group of **8a** remained in-

**Figure 31.****Figure 32.**

tact (Figure 29).⁴⁶ The success was achieved (Figure 30) by hydrolytic diazotization of the stereoisomeric 2,6-diaminopurine pronucleotides **99c**⁵³ using the protocol (Figure 19) elaborated for methylenecyclopropane analogues **8a** and **9a** (Figure 30). In the pyrimidine series, thymine pronucleotides **12b** and **13b** were prepared by a direct phosphorylation of synthymol (**1d**) or the *E*-isomer **2d** with reagent **101** (Figure 31). For synthesis of cytosine analogues **12a** and **13a**, N^4 -benzoyl derivatives **103a** and **103b**⁵¹ obtained as intermediates in resolution of the *Z*- and *E*-isomers **1c** and **2c** served as starting materials.¹ Compound **103a** or **103b** was phosphorylated with reagent **101** to give phosphoramidate **104a** or **104b** (Figure 32).

N-Debenzoylation was effected with hydrazine in pyridine–acetic acid buffer³⁵ furnishing pronucleotides **12a** and **13a**. The reactions described in Figures 30 and 32 indicate that synthetic operations can be successfully performed in the presence of a sensitive phenyl phosphoralaninate grouping if a use of basic reagents is avoided.

In the second-generation series, selective phosphorylation of a single hydroxy group of analogues **4** and **5** was the key requirement for synthesis of analogues **14–19** listed in Figure 2. Thus, phosphitylation method used for the synthesis of synadenol triphosphate **105a**, the *E*-isomer **105b**⁷¹ and phosphorotryptophanyl pronucleotides **106a**, **106b**

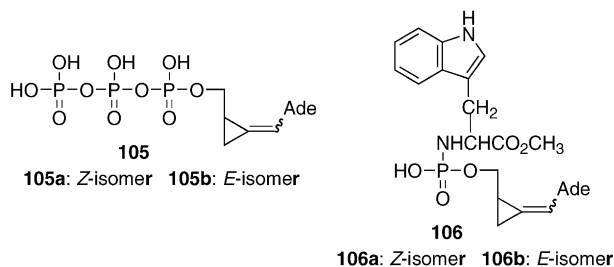


Figure 33.

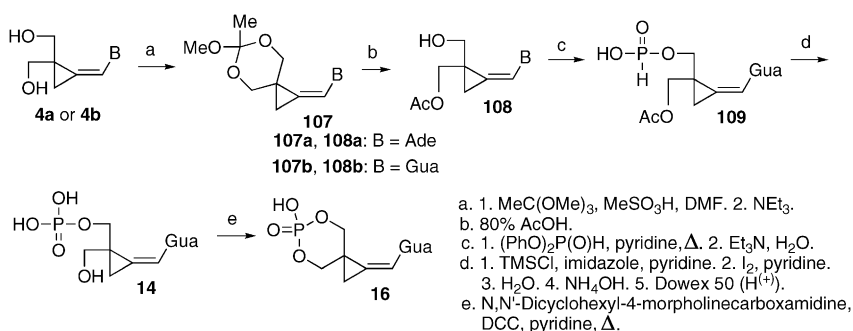
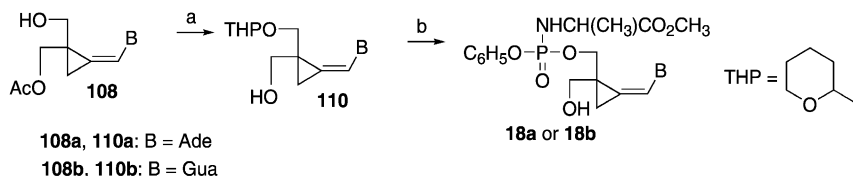


Figure 34.

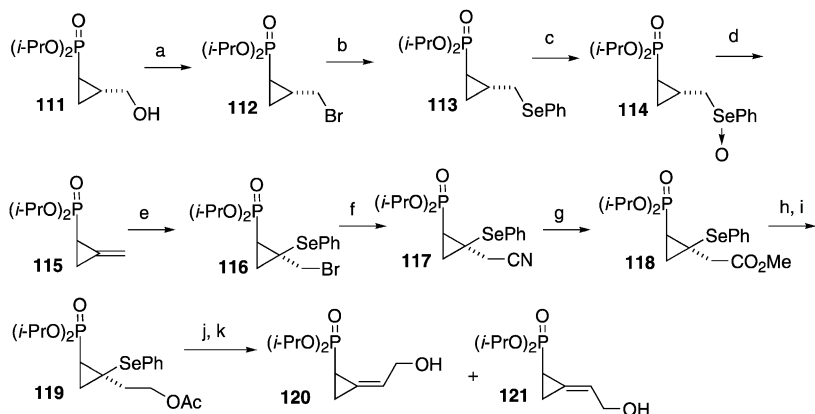
(Figure 33)⁶⁹ was applied for preparation of cyclopropavir phosphate **14** and the corresponding *E*-isomer **15**⁷³ (Figure 34). Cyclopropavir (**4b**) was first converted to the cyclic orthoacetate **107** which was hydrolyzed *in situ* with 80% acetic acid to give monoacetate **108**. Reaction with diphenyl phosphite afforded phosphite **109** which, after oxidation with iodine and deacetylation, furnished phosphate **14**. In a similar fashion, the *E*-isomer **5b** was transformed to phosphate **15**. Cyclization of **14** or **15** by a procedure described for ribonucleoside 3',5'-cyclic phosphates⁶⁰ afforded cyclic phosphates **16** or **17**.

For the synthesis of pronucleotide⁷³ **18a** or **18b**, acetates **108a** or **108b** could not serve as starting materials because removal of the acetyl group is not compatible with the base-sensitive phenyl phosphoralaninate function. By contrast, pronucleotides such as **18** or **19** are stable in acid media⁵² (Figure 30). Therefore, acetates **108a** and **108b** were transformed to the tetrahydropyranyl derivatives **110a** and **110b** (Figure 35). Phosphorylation with reagent **101** (see Figure 29) gave after deprotection in 80% acetic acid pronucleotides **18a** and **18b**.



- a. 1. Dihydropyran, MeSO_3H , DMF. 2. NH_4OH , Δ .
 b. 1. **101**, 1-methylimidazole, pyridine/THF. 2. 80% AcOH. 3. 0.1 Na_2HPO_4 , pH 7.5, MeOH.

Figure 35.



- a. Ph_3P , Br_2 , CH_2Cl_2 . b. 1. $(\text{PhSe})_2$, EtOH. 2. NaOH, NaBH_4 . c. H_2O_2 , THF. d. $(i\text{-Pr})_2\text{NET}$, toluene, Δ .
 e. $(\text{PhSe})_2$, NBS, CH_2Cl_2 . f. Me_3SiCN , Bu_4NF , MeCN. g. HCl(g) , MeOH. h. LiBH_4 , THF. i. Ac_2O , pyridine.
 j. 1. H_2O_2 , THF. 2. Δ . k. K_2CO_3 , MeOH/ H_2O .

Figure 36.

The *E*-isomers **19a** and **19b** were obtained along similar lines from the parent analogues **5a** and **5b**.

E. Phosphonate analogues **20a**, **20b**, **21a** and **21b**

The synthesis commenced with diisopropyl *trans*-(hydroxymethyl)-cyclopropyl phosphonate (**111**, Figure 36)²¹ which was transformed to bromo derivative **112**.⁷⁴ Phenylselenenylation (**113**) followed by oxidation (**114**) and elimination gave methylenecyclopropane phosphonate **115**. The *E* stereochemistry of the starting phosphonate **111** is not essential because it was abolished in the sequence leading to **115**. Bromoselenenylation of **115** (see Figure 27) was stereoselective giving

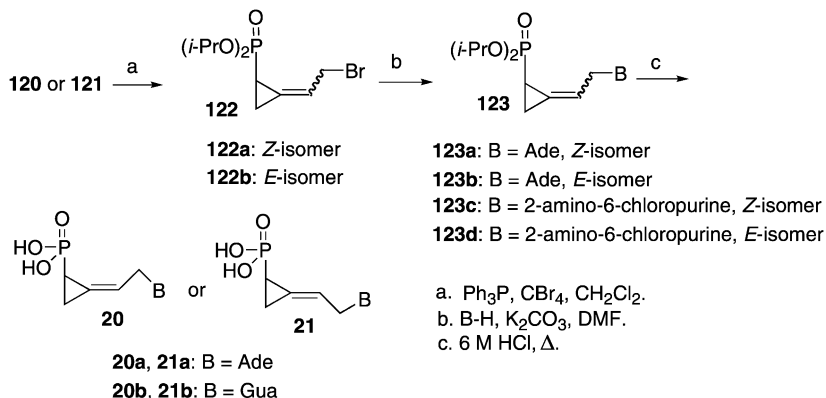


Figure 37.

a single isomer **116**. Substitution of bromine with cyanide was effected using Me_3SiCN and NBu_4F reagent⁶¹ to give nitrile **117** which was converted by methanolysis to ester **118**. Reduction with LiBH_4 followed by acetylation afforded acetate **119**. Oxidation with H_2O_2 gave directly the *Z*- and *E*-methylenecyclopropanes **120** and **121** (ratio 1 : 1) which were separated by chromatography. Also, the *Z* stereochemistry which was carried from compound **116** through **119** was abolished. Individual isomers **120** and **121** were transformed to bromo derivatives **122a** and **122b** which served for alkylation of adenine to furnish intermediates **123a** and **123b** (Figure 37). In a similar fashion, alkylation of 2-amino-6-chloropurine afforded compounds **123c** and **123d**. Acid hydrolysis of **123a** and **123b** gave phosphonate analogues **20a** and **21a**. In case of **123c** and **123d**, a simultaneous hydrolytic removal of isopropyl groups and chlorine was achieved to give guanine analogues **20b** and **21b**.

IV. ANTIVIRAL ACTIVITY

A. First-generation methylenecyclopropane analogues

The first-generation methylenecyclopropanes afforded a number of effective antiviral analogues.^{78,79} The SAR studies have gradually shifted from racemic to enantiomeric methylenecyclopropanes. Enantioselectivity of the antiviral effects of methylenecyclopropanes is quite complex^{78,79} and testing structurally novel analogues as racemates

Table 1. Inhibition of HCMV, EBV and VZV replication by 2-fluoro- and 2-chloropurine methylenecyclopropane analogues^a **1a**, **2a**, **6a**, **7a**, **6b** and **7b**

Compound	EC ₅₀ /CC ₅₀ (μM)				
	HCMV/HFF		EBV		VZV/HFF ^{d,h}
	Towne ^{b,c}	AD169 ^{d,e}	Daudi ^f	H-1 ^g	
1a	2.1/>100	1.3/>460	3.2/368 ⁱ	0.2/>50	2.5
2a	>100/>100	>460/>460	71.4/>229 ⁱ	3/50	90.2
6a	31/10	>4/12.8	>50/>50	>20/8.5	>4
7a	3.5/10	>0.8/1.5	>50/>50	>20/1.4	>0.8
6b	>100/>100	>60/>300	<0.08/>50	>20/>100	>60
7b	>100/>100	231/>300	<0.08/>50	>20/>100	179
Control	1.7/>100 ^j	0.22/40 ^j	0.93 ^k	5 ^j	0.22 ^k

^aData were taken from Qin et al.⁴⁶ and Qiu et al.^{50,52} (**1a**, **2a**).^bPlaque reduction assay.^cVisual cytotoxicity in stationary cells.^dCytopathic effect (CPE) inhibition assay.^eCytotoxicity by neutral red uptake.^fViral capsid antigen (VCA) ELISA.^gDNA hybridization assay. Cytotoxicity was determined in CEM cells.^hFor cytotoxicity see HCMV(AD169)/HFF.ⁱViral capsid antigen immunofluorescence (VCA-IF) assay.^jGanciclovir.^kAcyclovir.

first is a preferable option. New series of racemic analogues⁴⁶ include 2-fluoro- and 2-chloropurine methylenecyclopropanes **6a**, **7a**, **6b** and **7b** (Figure 2). A comparison of their antiviral activity with the corresponding non-fluorinated analogues, synadenol (**1a**) and the *E*-isomer **2a** is shown in Table 1. None of these analogues have reached the anti-CMV potency and low cytotoxicity of the parent compound synadenol (**1a**). The 2-fluoropurine *Z*- and *E*-methylenecyclopropanes **6a** and **7a** are cytotoxic and this effect offsets their antiviral activity. Interestingly, both analogues were inactive but not cytotoxic in EBV/Daudi culture. The corresponding chloro analogues **6b** and **7b** were non-cytotoxic but inactive with exception of EBV in Daudi culture, where both compounds were equipotent at submicromolar level (EC₅₀ < 0.08 μM) with a selectivity index of >625. Nevertheless, this effect was not reproduced in H-1 cells where no noticeable inhibitory activity was found. Similar differences between both assays were found for syncytol (**1c**) and its *E*-isomer **2c**⁵¹ and they may reflect differences in assay methodology (VCA-IFA or ELISA *vs* DNA hybridization) and/or type of the

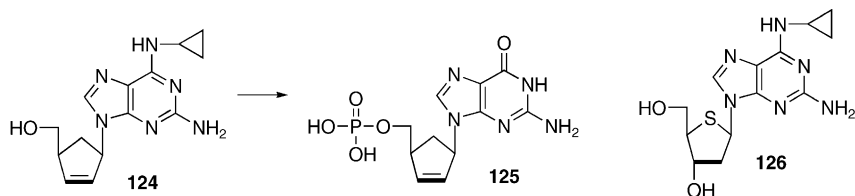


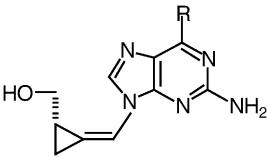
Figure 38.

cells (Daudi *vs* H-1). Thus, analogues **1c** and **2c** were also equipotent with $EC_{50} < 0.41 \mu\text{M}$ by VCA-IFA in Daudi cells, whereas DNA hybridization indicated EC_{50} 19.7 and $>50 \mu\text{M}$, respectively. A similar assay in H-1 cells indicated $EC_{50} > 50 \mu\text{M}$ for both compounds. Enantiomeric isoguanine analogues **8a** and **9a** were inactive.⁴⁶

The first-generation enantiomeric analogues have shown striking differences in enantioselectivity patterns between different viruses (HCMV *vs* EBV) and even between host cells or assay types (EBV). In HCMV and MCMV assays, the 2-aminopurine methylenecyclopropanes are strictly *S*-selective as established for the series **1b–1g** and **3** (Table 2).⁵³ The adenine analogues **S-1a** and **R-1a** are an exception because they are equipotent or almost equipotent against HCMV but they exhibit an *S*-selectivity for murine virus.⁴⁹ For these reasons, the fine-tuning studies of the 2-aminopurine 6 substituent were performed only with *S*-enantiomers **65a–65o**.^{10,31} It is clear that lipophilic substituents are compatible with a high anti-CMV potency. A heteroatom in the purine 6 position (N, O or S) does not seem to play a significant role as long as the attached alkyl does not carry a polar group (compound **65g** was less potent). Whereas longer aliphatic chains (pentyl of **65j** and **65m**) are tolerated, the bulkier substituents such as benzyl or cyclohexyl have a detrimental effect (**65e** and **65f**). Interestingly, the lipophilic threshold of activity has not been reached with pentyl substituents of **65j** and **65m**. It cannot be excluded that all active 2-aminopurine methylenecyclopropanes listed in Table 2 are prodrugs of synguanol (**S-1b**). A similarity of 2-amino-6-cyclopropylaminopurine analogue **3** with anti-HIV drug abacavir (**124**), which is a prodrug^{13,16} of carbovir 5'-phosphate (**125**, Figure 38) is intriguing. The antiviral agent 2-amino-6-cyclopropylaminopurine 2'-deoxy-4'-thioriboside (**126**) and related compounds⁶⁶ may then represent another analogy with the series shown in Table 2.

The 6-deoxypurine analogue **65o** which is largely devoid of *in vitro* antiviral activity⁷⁰ is oxidized by xanthine oxidase to synguanol (**S-1b**,

Table 2. Inhibition of human and murine cytomegalovirus (HCMV and MCMV) replication by enantiomeric methylenecyclopropane analogues of purine nucleosides^a

				
Compound	R	EC ₅₀ (μM)		
		HCMV/HFF ^b		MCMV/MEF ^b
		Towne ^c	AD169 ^d	
S-1a	NH ₂ , 2-NH ₂ = H	2.4	1.9	0.55
R-1a	NH ₂ , 2-NH ₂ = H	2.5	6.9	55
S-1b	OH	2.5	2.6	0.39
R-1b	OH	>100	>429	>429
S-1e	Cl	1.8	1.9	—
R-1e	Cl	30/32	256	—
S-1f	NH ₂	21	18	0.65
R-1f	NH ₂	>100	>431	>86
S-1g	OCH ₃	2.5	2.0	0.24
R-1g	OCH ₃	>100	252	32
S-3	CpNH ^e	1.8	1.9	0.15
R-3	CpNH ^e	>100	>74	44
65a	CH ₂ =CH-CH ₂ NH	1.8	2.8	0.33
65b	CH≡CH-CH ₂ NH	3.5	2.6	0.37
65c	CpCH ₂ NH ^e	3.8	52	1.7
65d	(CH ₃) ₂ CHNH	3.8	>365 ^f	not tested
65g	HO(CH ₂) ₂ NH	39	>72	11.9
65h	CH ₂ =CH-CH ₂ O	1.2	2.1	0.29
65i	CH ₃ (CH ₂) ₂ O	0.40	2.0	<0.11
65j	CH ₃ (CH ₂) ₄ O	0.21	1.4	1.45
65k	CpCH ₂ O ^e	0.32	1.7	0.35
65l	CH ₃ (CH ₂) ₂ S	0.22	3.0	1.4
65m	CH ₃ (CH ₂) ₄ S	0.32	1.5	1.5
65n	SH	37	45	>16/64
Ganciclovir		4.1	5.1	2.1

^aData were taken from Qiu et al.^{49,53} and Chen et al.¹⁰ Inactive *S*-enantiomers **65e**, **65f** and **65o** (EC₅₀ >100 μM) are not listed. The CC₅₀ values were >100 μM.

^bPlaque reduction assay.

^cVisual cytotoxicity.

^dCytotoxicity by neutral red uptake.

^eCp = cyclopropyl.

^fCytopathic effect (CPE) inhibition assay.

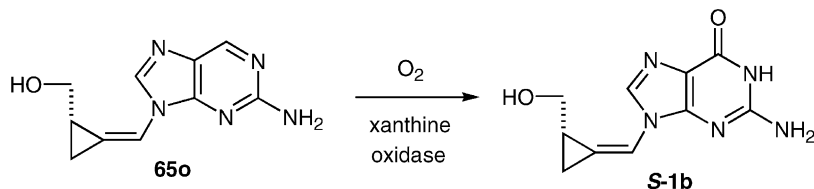


Figure 39.

Figure 39). The 6-deoxypurine congeners of guanine-based antivirals such as acyclovir and penciclovir are also substrates for this enzyme.^{22,30} The inactive analogue **65o** may then serve *in vivo* as a potential prodrug of synguanol (**S-1b**).

In vivo studies of purine methylenecyclopropane analogues were first performed in BALB/c mice infected with MCMV either intraperitoneally (i.p.) or orally⁵⁷ with racemic compounds. Synadenol (**1a**) and synguanol (**1b**) were effective after i.p. administration as the control (ganciclovir) but not at lower concentrations. After oral treatment, the activity of tested analogues followed roughly the pattern synadenol (**1a**) < (synguanol (**1b**) < 2-amino-6-methoxypurine analogue (**1g**) < 2-amino-6-cyclopropylaminopurine analogue **rac-3** (QYL-769). The potency of **rac-3** coincided with that of ganciclovir. Studies of pathogenesis of MCMV infection indicated reduction of the virus titer in several BALB/c mice organs with comparable effects with both **rac-3** and ganciclovir but neither of the drugs was able to completely clear the virus from the body. On the basis of these results, the *S*-enantiomer **3** (QYL-1064)⁴ was then selected for additional studies. BALB/c mice infected with MCMV and SCID mice implanted with human fetal retinal or thymus/liver tissue inoculated with Toledo strain of HCMV were used. In all these experiments, the *S*-enantiomer **3** was comparable or somewhat better than ganciclovir.

The EBV results have presented a more complex picture (Table 3). Enantiomeric adenine analogues were both effective in all three assays but the *R*-enantiomer **R-1a** was more potent than *S*-enantiomer **S-1a**.⁴⁹ The strict *S*-enantioselectivity observed with 2-aminopurine methylenecyclopropanes for HCMV or MCMV (Table 2) was absent. Only synguanol (**1b**) exhibited an *S*-selectivity with **S-1b** about 17–87 times more potent than **R-1b**.⁵³ Analogues **1e**, **1f** and **1g** had a diminished enantioselectivity. The (*S*)-2-amino-6-cyclopropylamino analogue **3** was more effective than the *R*-enantiomer **R-3** in all three assays. In the *S*-series **65a–65e**, **65h–65n** (the *R*-enantiomers of compounds

Table 3. Inhibition of Epstein–Barr virus (EBV) replication by enantiomeric methylenecyclopropane analogues of purine nucleosides^a

Compound	R	EC ₅₀ (μM) ^b		
		EBV/ Daudi ^c		EBV/H-1 ^d
		VCA	DNA hybridization	
S-1a	NH ₂ , 2-NH ₂ =H	7.8	1	0.63
R-1a	NH ₂ , 2-NH ₂ =H	3	<0.37	0.09
S-1b	OH	2.3	0.34	0.47
R-1b	OH	40	29	41
S-1e	Cl	1.6	0.76	1.1
R-1e	Cl	5.2	6.4	27
S-1f	NH ₂	0.69	60	1.3
R-1f	NH ₂	12	12	2.3
S-1g	OCH ₃	3.8	1.0	0.44
R-1g	OCH ₃	1.3	5.3	15
S-3	CpNH ^e	6.2	0.85	0.66
R-3	CpNH ^e	61	1.3	49

(continued on next page)

listed in Figure 18 have not been synthesized), differences between the cell and assay types became more pronounced.^{10,31} Thus, the anti-EBV activity at micromolar level or below was observed with analogues **65b**, **65h–65j** and **65l** but only in H-1 cells. With the exception of 2-amino-6-cyclopropylmethylamino analogue **65c**, they were all inactive or less potent in Daudi culture. The observed differences can be explained by assuming different activation (phosphorylation) mechanisms in different host cells.

Antiviral activity was also noted with herpesviruses sharing a genomic similarity with HCMV or EBV. The results with a series of analogues **3**, **65a–65c** and **65h–65m** against β -herpesvirus HHV-6 (two strains) and γ -herpesvirus HHV-8 are summarized in Table 4.³¹ A good correlation with CMV results (Table 2) was observed in both strains of HHV-6. An increased cytotoxicity was seen in several cases in HSB-2 cells. The 2-amino-6-cyclopropylmethylaminopurine analogue **65c** was the only compound which was inactive against both strains of HHV-6.

Table 3. — Continued

Compound	R	EC ₅₀ (μM) ^b		
		EBV/ Daudi ^c		EBV/H-1 ^d
		VCA	DNA hybridization	
65a	CH ₂ =CH-CH ₂ NH	>184	—	18.5
65b	CH≡CH-CH ₂ NH	>185	—	~2
65c	CpCH ₂ NH ^e	0.6	7	>20
65d	(CH ₃) ₂ CHNH	>182	—	11
65e	C ₆ H ₅ CH ₂ NH	52	—	>20
65h	CH ₂ =CH-CH ₂ O	>183	—	1.1
65i	CH ₃ (CH ₂) ₂ O	>182	—	1.5
65j	CH ₃ (CH ₂) ₄ O	23.4	—	7.3
65k	CpCH ₂ O ^e	29.2	—	12.6
65l	CH ₃ (CH ₂) ₂ S	>172	—	6.3
65m	CH ₃ (CH ₂) ₄ S	39.8	—	>20
65n	SH	>8 ^f	—	>20
Control		1.1 ^g	—	5 ^h

^aThe results were taken from Qiu et al.^{49,53}, Chen et al.¹⁰ and Kushner et al.³¹ Compounds **65f**, **65g** and **65o** with EC₅₀ > 100 μM in Daudi cells (both assays) and EC₅₀ > 20 μM in H-1 culture are not listed.

^bThe CC₅₀ values which were >100 μM (Daudi cells) and >20 μM (H-1 cells) for all tested compounds are not listed.

^cViral capsid antigen immunofluorescence (VCA-F) assay for compounds **S-1a** through **R-3** and VCA-ELISA for analogues **65a** through **65o**.

^dCytotoxicity was determined in CEM cells.

^eCp = cyclopropyl.

^fCC₅₀ 35.3 μM.

^gAcyclovir.

^hGanciclovir.

By contrast, the results with HHV-8 correlated poorly with those obtained in EBV assays although somewhat better correspondence was observed in H-1 culture than Daudi cells. At any rate, all tested compounds with the exception of analogue **65c** were more effective than the cidofovir control.

In general, methylenecyclopropane analogues are less active against α-herpesviruses. In HFF cells infected with HSV-1 or HSV-2, the 2-

Table 4. Inhibition of human herpes virus 6 (HHV-6A and HHV-6B) and human herpes virus 8 (HHV-8 replication by (*S*, *Z*)-2-amino-6-substituted purine methylenecyclopropanes^a

Compound	R	EC ₅₀ /CC ₅₀ (μM)		
		HHV-6A/HSB-2	HHV-6B/CBL	HHV-8/BCBL-1

S-3	CpNH ^b	3.3/25.6	1.6/>178	1.5/110
65a	CH ₂ =CH-CH ₂ NH	8.3/>184	3.6/>184	4.1/>184
65b	CH≡CH-CH ₂ NH	100/162	5.4/>185	6.5/>185
65c	CpCH ₂ NH ^b	108/>175	90/>175	>175/>175
65h	CH ₂ =CH-CH ₂ O	3.8/46	<1.1/>165	2.9/118
65i	CH ₃ (CH ₂) ₂ O	1.1/26	1.0/>162	1.1/>182
65j	CH ₃ (CH ₂) ₄ O	2.3/>96	1.2/>99	1.2/109
65k	CpCH ₂ O ^b	3.1/54	1.2/138	2.6/130
65l	CH ₃ (CH ₂) ₂ S	9.6/61	3.0/>148	3.7/148
65m	CH ₃ (CH ₂) ₄ S	16.8/>156	3.4/>156	4.2/>156
Cidofovir		3.1/>150	1.0/>132	12.8/>154

HSB-2 = human T-cell lymphoblastoid cell line, CBL = cord blood lymphocytes, BCBL-1 = body cavity-based lymphoma.

^aData were taken from Kushner et al.³¹

^bCp = cyclopropyl.

amino-6-cyclopropylmethoxypurine analogue **65k** was the only effective analogue (EC₅₀ 6.3 μM) whereas the rest of the tested group was either inactive or lacked a significant potency.³¹ Nevertheless, more activity was seen in HSV-1/BSC-1 culture (ELISA) or HSV-1 and HSV-2 in Vero cells. Table 5 lists only analogues with EC₅₀ in micromolar range in all three assays.¹⁰ A similar situation was encountered in VZV assays in HFF culture (Table 6).^{10,31} Again, only a few methylenecyclopropane analogues exhibited potent activity, although the results did not exactly parallel those obtained with HSV-1 and HSV-2 (Table 5). The purine O⁶-alkyl derivatives **65h**, **65i** and **65j** were the most efficacious in both cytopathic and plaque reduction assays.

Against non-herpesviruses such as HIV-1 or HBV, the antiviral activity of methylenecyclopropanes was only sporadic and relatively

Table 5. Inhibition of herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) replication by selected (*S*, *Z*)-2-amino-6-substituted purine methylenecyclopropanes^a

Compound	R	EC ₅₀ /CC ₅₀ (μM)		
		HSV-1/BSC-1 ^b	HSV-1/Vero ^{c,d}	HSV-2/Vero ^{d,e}
65h	CH ₂ =CH-CH ₂ O	1.5	7/26	8
65i	CH ₃ (CH ₂) ₂ O	1	4/32	10
65j	CH ₃ (CH ₂) ₄ O	7	3/87	4
65k	CpCH ₂ O ^f	7	8/41	12
65l	CH ₃ (CH ₂) ₂ S	1	9/>100	10
Acyclovir		0.15	13.5/>200	32.3

^aData were taken from Chen et al.¹⁰^bELISA results, EC₅₀ data only, CC₅₀'s in growing KB cells were >100 μM in all cases.^cPlaque reduction assay.^dCytotoxicity was determined in CEM cells.^eEC₅₀ data only, for CC₅₀'s see HSV-1.^fCp = cyclopropyl.

moderate. Thus, The only first-generation methylenecyclopropane analogue found effective against HIV-1 is *R*-synadenol (**R-1a**) with EC₅₀ 11 μM in CEM-SS culture⁴⁹ and 13 μM in MT-2 cells.¹¹ The latter reference reported an erroneous configurational assignment for *R*- and *S*-enantiomers of synadenol (**1a**). The enantiomer **S-1a** was devoid of anti-HIV activity. Adenallene (**22a**) which can be regarded as an analogue of synadenol (**1a**, Section II, Figure 3) was a more powerful anti-HIV agent but exhibited the same enantiomeric preference; the *R*-enantiomer being active, whereas the *S*-enantiomer was devoid of potency.³⁸

Several methylenecyclopropane analogues were found moderately effective against HBV in 2.2.15 culture (Table 7) with less pronounced enantioselectivity. Thus, both enantiomers of adenine and 2,6-diaminopurine analogues **1a** and **1f** were effective with some *S*-selectivity.^{49,53} Active compounds **65h–65l** were tested only as *S*-enantiomers¹⁰ and **65l** was the least cytotoxic of this group of analogues.

Table 6. Inhibition of varicella-zoster virus (VZV) replication by selected (*S, Z*)-2-amino-6-substituted purine methylenecyclopropanes^a

Compound	R	VZV/HFF, EC ₅₀ (μM)
S-3	CpNH ^b	7.0 (12)
65c	CpCH ₂ NH ^b	10 (10.8)
65h	CH ₂ =CH-CH ₂ O	1.1 (2.3)
65i	CH ₃ (CH ₂) ₂ O	0.5 (8)
65k	CpCH ₂ O ^b	0.5 (8.4)
65m	CH ₃ (CH ₂) ₄ S	2.3 (12.5 ^c)
Acyclovir		2.1 (1.5)

^aData were taken from Kushner et al.³¹ (CPE data) and Chen et al.¹⁰ (plaque reduction data listed in parentheses). The CC₅₀'s were in all cases >300 μM.

^bCp = cyclopropyl.

^cCorrected value.

B. Second-generation methylenecyclopropane analogues

This series of compounds included the *Z*- and *E*-isomers derived from four DNA bases **4a**, **5a**, **4b**, **5b**, **4c**, **5c**, **4d**, **5d**, the purine 6-substituted analogues **4e**, **5e**, **4f**, **5f**, **4g**, **5g** (Figure 1) and **73a**, **74a**, **73b**, **74b** (Figure 21). In terms of antiviral effects, these analogues have a more narrow range of potency but they have yielded the most effective anti-HCMV agent of the methylenecyclopropane group of analogues, cyclopropavir (CPV, **4b**).^{28,82} The lack of chirality of these compounds is an obvious advantage.

The in vitro anti-CMV activity of these analogues is summarized in Table 8. CPV (**4b**) was clearly the most potent compound in all three assays. Surprisingly, 2-amino-6-cyclopropylamino analogue **73a** was inactive although similar first-generation congener **3** is a strongly potent agent (Section A). Consequently, it cannot function as a prodrug of CPV (**4b**). By contrast, such a mechanism cannot be a priori excluded for the second most potent analogue of this series, 2-amino-6-methoxypurine compound **4g**. Adenine congener **4a** was more effective than the cytosine analogue **4c**, but the 2,6-diaminopurine compound **4f** was potent only in the Towne strain of HCMV. As in the first-generation series,

Table 7. Inhibition of hepatitis B virus (HBV) replication by selected (S, Z)-(+)-2-amino-6-substituted purine methylenecyclopropanes^a

Compound	R	HBV/2.2.15 ^{b,c} , EC ₅₀ /CC ₅₀ (μM)
S-1a	NH ₂ , NH ₂ = H	9/65
R-1a	NH ₂ , NH ₂ = H	16/>100
S-1f	NH ₂	5/>100
R-1f	NH ₂	10/>100
65h	CH ₂ =CH-CH ₂ O	11/26
65i	CH ₃ (CH ₂) ₂ O	4.1/32
65j	CH ₃ (CH ₂) ₄ O	7.3/87
65k	CpCH ₂ O ^d	12.6/41
65l	CH ₃ (CH ₂) ₂ S	5.1/>100
Lamivudine		0.02/>100 ^e

^aData from Qiu et al.^{49,53} and Chen et al.¹⁰^bDNA hybridization assay.^cCytotoxicity was determined in CEM cells.^dCp = cyclopropyl.^eLamivudine (3TC).

the *E*-isomers were inactive or they exhibited at best a moderate effect (**5b**). The *Z*- and *E*-isomeric fluoro analogues **73b** and **74b** were roughly equally effective but modest inhibitors. Absence of cytotoxicity is a hallmark of this series of analogues.

Because of a potent in vitro activity, CPV (**4b**) was selected for in vivo studies in mice models of HCMV infection and the results were compared with current drug ganciclovir (GCV, **30**).²⁷ In immunocompetent BALB/c mice infected with MCMV orally administered CPV (**4b**) significantly reduced mortality at 3 mg and 10 mg/kg, while GCV (**30**) was effective only at 10 mg/kg. Similar treatment of severe combined immunodeficient (SCID) mice CPV (**4b**) reduced the MCMV titer in organs more effectively than GCV (**30**) by 2 to 5 log₁₀. The HCMV models included SCID mice with implanted human fetal retinal or thymus/liver tissue infected with HCMV. In both cases, cyclopropavir (**4b**) was more effective than GCV (**30**) in reducing the virus titer to un-

Table 8. Inhibition of human and murine cytomegalovirus (HCMV and MCMV) replication by the 2,2-bis(hydroxymethyl)methylenecyclopropane analogues^a

Compound	EC ₅₀ /CC ₅₀ (μM)			
	Configuration/B	HCMV/HFF ^b		MCMV/MEF ^b
		Towne ^c	AD169 ^d	
4a	Z/Ade	3.6/100	11.7/>404	9.7/>404
4b	Z/Gua	0.46/>100	0.49/>380	0.27/>380
5b	E/Gua	39/>100	123/>380	42/>380
4c	Z/Cyt	32/>100	14.3/>448	54/>448
4f	Z/DAP	15/>100	>381/>381 ^e	not tested
4g	Z/AMP	3.5/>100	2.7/>361	2.0/>361
73b	Z/AFP	38/>100	22.2/>377	8.7/>377
74b	E/AFP	33/>100	43.7/>377	18.9/176
Ganciclovir		4.1/>100	2.3/>392	5.0/>35

^aThe results were taken from Zhou et al.⁸² Compounds inactive in both Towne and AD169 strains of HCMV are not listed. They were not tested against MCMV. DAP = 2,6-diaminopurine, AMP = 2-amino-6-methoxypurine and AFP = 2-amino-6-fluoropurine.

^bPlaque reduction assay.

^cVisual cytotoxicity.

^dCytotoxicity by neutral red uptake.

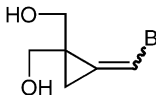
^eCytopathic effect (CPE) inhibition assay.

detectable levels. All these results indicate that a further preclinical evaluation CPV (**4b**) is warranted.

Not surprisingly, significant in vitro effects against replication of EBV were also observed (Table 9).^{28,82} The most active analogues in Daudi cells were the *E*-isomers **5f** and **74a**, although the EC₅₀'s were higher in DNA hybridization assay and no significant potency was recorded in H-1 culture. Cyclopropavir (**4b**) was only effective in H-1 cells, whereas the thymine analogue **4d** exhibited significant activity in Daudi cells but it was only a moderate inhibitor in H-1 cells. By contrast, the *Z* isomer of 2-amino-6-methoxypurine analogue **4g** was the most potent compound in the H-1 culture but it was inactive in Daudi cells. Similar dependence of anti-EBV potency on the type of cells and assays was observed in the first-generation series (Section A).

The second-generation methylenecyclopropane analogues were also effective against HHV-6 and HHV-8. The active compounds are listed

Table 9. Inhibition of EBV replication by the 2,2-bis(hydroxymethyl)methylenecyclopropane analogues^a

			
Compound	EC ₅₀ /CC ₅₀ (μM)		
	Configuration/B	EBV/Daudi ^b	EBV/H-1 ^{c,d}
4a	Z/Ade	166/>202	16/>50
4b	Z/Gua	45/74	7/>50
4d	Z/Thy	4.6/>210	19/>50
4e	Z/ACP	>178/>178	16/24
5e	E/ACP	142/>178	16/12
5f	E/DAP	<0.31/>191(15.3) ^c	>20/>100
5g	Z/AMP	161/>180	4.8/90
73b	Z/AFP	>189/>189	20.8/31
74a	E/ACAP	0.30/>165 (19.9) ^c	>20/>100
74b	Z/AFP	>189/>189	18/56
Control		1.1/>222 ^e	5 ^f

^aData from Zhou et al.⁸² Compounds inactive in both assays with EC₅₀ >100 (Daudi) and >20 (H-1) are not listed. For abbreviations, see Table 8. ACAP = 2-amino-6-cyclopropylaminopurine.

^bViral capsid antigen (VCA) ELISA.

^cDNA hybridization assay.

^dCytotoxicity was determined in CEM cells.

^eAcyclovir.

^fGanciclovir.

in Table 10.²⁸ Interestingly, cyclopropavir (**4b**) was efficacious in all three assays. The activity of other analogues varied. Thus, thymine derivative **4d** was active against HHV-8 and 2-amino-6-methoxypurine compound **4g** inhibited the HHV-6B strain but to a lesser extent HHV-6A and HHV-8. Analogue **4a** was a potent inhibitor of HHV-6B.

The 2-amino-6-methoxypurine methylenecyclopropane **4g** was the only analogue effective against VZV (plaque reduction assays, EC₅₀ 3 μM) whereas 2,6-diaminopurine **4f** inhibited replication of HBV (EC₅₀ 4 μM).⁸²

C. Antiviral activity of fluorinated methylenecyclopropane analogues

Geminal methylenedifluorocyclopropanes **79a**, **79b**, **80a** and **80b** (Figure 23) did not exhibit any significant antiviral potency. Only *gem*-

Table 10. Inhibition of HHV-6 and HHV-8 replication by selected 2,2-bis(hydroxymethyl)methylenecyclopropane analogues^a

Compound	B	EC ₅₀ (μM) ^b		
		HHV-6A/HSB-2	HHV-6B/CBL	HHV-8/BCBL-1
4a	Ade	18	4.6	57
4b	Gua	7.8	0.7	6.5
4c	Cyt	18	23	197
4d	Thy	not tested	not tested	5.5
4g	AMP	28	2.3	16
Cidofovir		5.7	1.4	0.4 ^c

^aData from Kern et al.²⁸ AMP = 2-amino-6-methoxypurine. For abbreviations of the host cells see Table 4. AMP = 2-amino-6-methoxypurine.

^bThe CC₅₀ was >360 μM in all cases as determined in HFF culture.

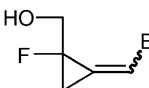
^cGanciclovir.

difluorosynadenol (**79a**) was moderately active against HCMV.⁶⁸ Selected compounds from the group of monofluorinated analogues **10a–10d** and **11a–11d** (Figure 2) were effective against several viruses.⁸³ Adenine *Z*-isomer **10a** was significantly more effective against HCMV in HFF culture than the difluoro congener **79a** with EC₅₀/CC₅₀ in Towne strain of virus 3.6/>100 μM and in AD169 strain 6.0/>425 μM (plaque reduction), but EC₅₀/CC₅₀ in MCMV/MEF was only 69/>425 μM. The corresponding *E*-isomer **11a** had a borderline effect. The rest of the group was inactive including, somewhat surprisingly, the fluoro analogue of synguanol **10b**.

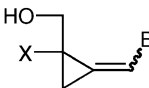
The EBV testing presented a different situation. Almost all analogues, *Z* and *E*, were effective at least in one assay (Table 11). Thymine *E*-isomer **11d** which was inactive in both Daudi and H-1 culture was the only exception. The results reinforce the conclusion that the *E*-isomerism is no severe obstacle to anti-EBV activity of methylenecyclopropanes in contrast to HCMV where the *Z*-isomers are the only potent analogues observed to date. Significant differences between type of assay and host cells were again observed.

Activity of fluoromethylenecyclopropanes against α-herpesviruses is limited to VZV (Table 11). The *Z*-isomers of pyrimidine analogues **10c** and **10d** were the most potent, whereas adenine *Z*- and cytosine *E*-

Table 11. Inhibition of EBV and VZV replication by (Z)- and (E)-2-(fluoro-2-hydroxymethyl)cyclopropyldenemethylpurines and -pyrimidines^a

				
Compound	Configuration/B	EC ₅₀ /CC ₅₀ (μM)		
		Daudi ^b	EBV H-1 ^{c,d}	VZV HFF ^e
10a	Z/Ade	1.45/> 213 (6.8)	13.8	14.0
10b	Z/Gua	4.8/> 199 (8.0)	>20	> 79.6
10c	Z/Cyt	<0.38/> 237 (28.4)	>20	0.62
10d	Z/Thy	51.7/> 221 (4.4)	2.5	0.62
11a	E/Ade	2.3/> 209 (167)	3.6	> 83.4
11b	E/Gua	<0.32/> 199 (29.1)	>20	> 79.6
11c	E/Cyt	0.76/> 237 (94.8)	>20	14.7
11d	E/Thy	> 221/> 221	>20	>442
Control		1.1/> 222 (5.3) ^f	5 ^g	1.6 ^f

^aData were taken from Zhou et al.⁸³^bViral capsid immunofluorescence (VCA) ELISA. Values in parentheses are for DNA hybridization assay.^cDNA hybridization assay.^dCytotoxicity was determined in CEM cells. The CC₅₀'s of all listed analogues were >100 μM.^eCytopathic effect (CPE) assay. The CC₅₀'s were >300 μM in all cases.^fAcyclovir.^gGanciclovir.**Table 12.** Comparison of anti-HIV activity of isomeric pairs of fluorinated and non-fluorinated analogues^a **10a**, **11a** and **1a**, **2a**

			
Compound	Isomer/X	EC ₅₀ /CC ₅₀	
		HIV-1/MT-2	HIV-1/MT-4
10a	Z/X = F	12/> 100	22/> 100
11a	E/X = F	2.3/> 100	7.6/> 100
1a	Z/X = H	0.75/32 ^b	–
2a	E/X = H	>100/> 100 ^b	–

^aData from Zhou et al.⁸³^bData from Uchida et al.⁶⁴

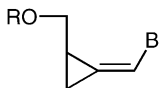
isomer **10a** and **11c** had a moderate effect. Inhibitory potency against other α -viruses was sporadic. Thus, the only analogue active against HSV-1 or HSV-2 was the thymine *Z*-isomer **11d** with an EC_{50} of 2.5 μ M in a single testing system HSV-1/BSC-1 cells (ELISA). No effect was found in either HFF or Vero culture. Quite surprisingly, the adenine *E*-isomer **11a** was more potent and less cytotoxic against HIV-1 than the *Z*-isomer **10a** (Table 12) whereas the opposite was true in case of synadenol (**1a**) and the *E*-isomer (**2a**). Against HBV, only a single analogue, guanine *Z*-isomer **11b**, was moderately effective with EC_{50} 15 μ M.

D. Pronucleotides and nucleotides of methylenecyclopropane analogues

Extension of the pronucleotide concept to methylenecyclopropane analogues led to several pronucleotides with high antiviral activity. These studies which mainly focused on phenyl phosphoralaninates were recently reviewed^{8,77} and, therefore, the emphasis will be put on recent results and some important generalizations. In general terms, introduction of the phenyl phosphoralaninate (PPA) grouping into the parent methylenecyclopropanes led in several cases to increases in antiviral activity although sometimes at the expense of elevated cytotoxicity. This potentiating effect was most clearly seen in HIV-1 and HBV assays. These results provided the first indication that phosphorylated intermediates are involved in the mechanism of action of methylenecyclopropane analogues. It should be emphasized that the parent methylenecyclopropanes are weak anti-HIV or anti-HBV agents and a circumvention of the difficult first phosphorylation step³⁷ may present a clear advantage (Table 13).^{52,64} Pronucleotides **99a** and **99c** (Figure 28) were very effective against both viruses, but the adenine analogue **99a** was more cytotoxic. With the 2,6-diaminopurine derivative **99c**, the best balance between antiviral effect and cytotoxicity was reached with selectivity index 190–618 (HIV-1) and 563 (HBV). Strong potentiating effect of PPA group was also evident in HIV-1 (353–500 fold) and HBV (125 fold) assays. Inactivity (HIV-1) or limited potentiating effect (HBV, factor of 5) of guanine pronucleotide **99b** suggests that other factors, e.g. heterocyclic base, may also influence the effect of PPA group on the antiviral activity of the parent methylenecyclopropane. Interestingly, even the *E*-isomer **100a** was effective with a potentiating factor of 77–>151 (HIV-1) and >8 (HBV).

By contrast, with methylenecyclopropanes strongly active against CMV which are effectively phosphorylated in infected host cells, such

Table 13. Comparison of inhibition of HIV-1 and HBV replication by selected phosphoralaninate prodrugs of methylenecyclopropane analogues with parent analogues^a

<div style="display: flex; align-items: center; justify-content: center;">  <div style="margin-left: 20px;"> $R = H \text{ or PPA, PPA} = \text{C}_6\text{H}_5\text{O}-\text{P}(=\text{O})(\text{NHCH}(\text{CH}_3)\text{CO}_2\text{CH}_3)-$ </div> </div>					
Compound	R	B	EC ₅₀ /CC ₅₀ (μM)		
			HIV _{III} RT ^b CEM-SS	HIV _{LAI} ^c MT-2	HBV/2.2.15 ^d
99a	PPA	Ade	0.05/1.1	0.0026/0.24	0.01/0.3 ^e
1a	H	Ade	20/>100	0.75/32	2/>100
99b	PPA	Gua	>100/>100	>76/76	2/>100
1b	H	Gua	>100/>100	>70/70	10/>100
99c	PPA	DAP	0.2/38	0.034/21	0.08/45 ^e
1f	H	DAP	>100/>100	12/>100	10/>100
100a^f	PPA	Ade	1.3/66	0.66/24	1.2/4.5 ^e
2a^f	H	Ade	>100/>100	>100/>100	>10/>100
Control			0.017 ^g	0.019/>100 ^g	1.4 ^h

^aData were taken from Qiu et al.⁵² and Uchida et al.⁶⁴ Abbreviations: DAP = 2,6-diaminopurine, AMP = 2-amino-6-methoxypurine, ACAP = 2-amino-6-cyclopropylaminopurine.

^bReverse transcriptase (RT) activity assay.

^cCytopathic effect (CPE) assay.

^dDNA hybridization assay. Cytotoxicity was determined in CEM cells (CC₅₀ >100 μM).

^eCytotoxicity was determined in 2.2.15 cells.

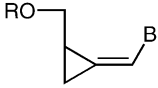
^f*E*-isomer.

^gAZT.

^hZalcitabine (ddC).

a clear-cut potentiating effect of PPA pronucleotides is rarely seen⁵² (Table 14). In contradistinction to HIV-1 and EBV, all PPA analogues listed are derived from strongly active parent analogues with the exception of **99c**. The anti-CMV activity trend then seems to indicate that when the parent analogue is less active the transformation to the PPA pronucleotide leads to an increase of potency. In the opposite case, the efficacy of the PPA pronucleotide either corresponds to the parent analogue or it is even lower. For example, potentiation of anti-CMV activity was found in pronucleotide **99c** derived from 2,6-diaminopurine analogue **1f**, less potent in HCMV assays. In MCMV culture, where **1f** is very active some decrease of efficacy was noted. The adenine analogue **99a** was too toxic to evaluate. The *E*-isomers **100a**, **100b**, **100c** and

Table 14. Comparison of inhibition of human and murine cytomegalovirus (HCMV and MCMV) replication by selected PPA pronucleotides and parent methylenecyclopropane analogues^a

<div style="display: flex; align-items: center; justify-content: space-around;"> <div style="text-align: center;">  </div> <div> $R = H \text{ or PPA, PPA} = \text{C}_6\text{H}_5\text{O}-\text{P}(=\text{O})(\text{NHCH}(\text{CH}_3)\text{CO}_2\text{CH}_3)-$ </div> </div>					
Compound	R	B	EC ₅₀ /CC ₅₀ (μM)		
			HCMV/HFF ^b		MCMV/MEF ^b
			Towne ^c	AD 169 ^d	
12a	PPA	Cyt	1.5/100	29.8/>300 ^e	not tested
1c	H	Cyt	28.5/>100	3.4/>518 ^e	–
99a	PPA	Ade	0.14/2.5	not tested ^f	not tested
1a	H	Ade	2.1/>100	–	–
99b	PPA	Gua	4.2/>100	25/>206	3.3/>206
1b	H	Gua	2.1/>100	1.2/>429	0.3/>429
99c	PPA	DAP	4/>100	7.8/121	3.2/211
1f	H	DAP	24/>100	16/266	0.6/293
99d	PPA	AMP	0.16/3.7	4.5/34	0.24/126
1g	H	AMP	3.1/>100	5.3/>404	0.4/261
99e	PPA	ACAP	0.25/10	6.2/126	0.94/101
rac-3	H	ACAP	0.4/>100	2.4/327	0.37/220
Ganciclovir			7.4/>100	0.78/>392	3.8

^aData were taken from Qiu et al.⁵² Data for **12a** and **1c** are from Ambrose et al.¹. Abbreviations: DAP = 2,6-diaminopurine, AMP = 2-amino-6-methoxypurine, ACAP = 2-amino-6-cyclopropylaminopurine.

^bPlaque reduction assay.

^cVisual cytotoxicity.

^dCytotoxicity by neutral red dye uptake.

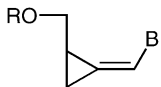
^eCytopathic effect (CPA) assay.

^fStrongly cytotoxic.

pyrimidine pronucleotides **12b**, **13a**, **13b** were inactive.^{1,52} The orally administered pronucleotide **99d** was about equally effective against MCMV infection in mice as was the parent analogue **1g**,⁵⁷ and little toxicity was observed.

The EBV and VZV in vitro results followed a pattern roughly similar to that found in CMV assays for the *Z*-isomers (Table 15),^{1,52} but the *E*-isomers **100a**, **100b** and **100c** were also effective in some EBV assays. Again, pronucleotides derived from potent analogues offered little tangible benefits over the parent compounds but activity of moderately effective methylenecyclopropanes was significantly increased

Table 15. Inhibition of Epstein–Barr virus (EBV) and varicella zoster virus (VZV) replication by PPA pronucleotides and parent methylenecyclopropane analogues^a

<div style="display: flex; align-items: center; justify-content: center;">  <div style="margin-left: 20px;"> $\text{R} = \text{H or PPA, PPA} = \text{C}_6\text{H}_5\text{O}-\text{P}(=\text{O})(\text{NHCH}(\text{CH}_3)\text{CO}_2\text{CH}_3)_2$ </div> </div>					
Compound	R	B	EC ₅₀ /CC ₅₀ (μM)		
			EBV/H-1 ^b	EBV/Daudi ^c	VZV/HFF ^d
12a	PPA	Cyt	16/74	0.65/>100 ^e	56.2/>300 ^f
1c	H	Cyt	2.5/>50	<0.41/>259 ^e	3.6/>518 ^f
99a	PPA	Ade	0.1/1 ^g	1.0/>108	7.6/4.3
1a	H	Ade	0.2/>50	3.2/368	2.3/>460
99b	PPA	Gua	>10/>50	0.31/>103	8.1/>206
1b	H	Gua	0.3/>50	5.6/>214	61/>429
99c	PPA	DAP	5.5/35 ^g	3.8/26	1.0/121
1f	H	DAP	1.5/>50	6.9/>215	93/266
99d	PPA	AMP	0.4/4.6	0.75/4.4	0.12/34
1g	H	AMP	1.6/>50	18/>202	24/>404
99e	PPA	ACAP	10/23	2.3/19	0.64/126
rac-3	H	ACAP	4/>100 ^e	12/>184	13/327
100a^h	PPA	Ade	4.0/40 ^e	3.2/65	111/141
2a^h	H	Ade	3/>50	71.4/>230	90/>460
100b^h	PPA	Gua	8/>50	>105/>105	>211/>211
2b^h	H	Gua	>50/>50	>206/>206	263/>413
100c^h	PPA	DAP	>10/>50	4.0/>106	not tested
2f^h	H	DAP	>50/>50	58/>215	–
Control			5 ⁱ	4.9/>222 ^j	9.3 ^j

^aData from Ambrose et al.¹ for **12a** and **1c** and Qiu et al.⁵² for the rest of analogues.^bDNA hybridization assay. Abbreviations: DAP = 2,6-diaminopurine, AMP = 2-amino-6-methoxypurine, ACAP = 2-amino-6-cyclopropylaminopurine.^cViral capsid antigen immunofluorescence (VCA-IF) assay.^dPlaque reduction assay. Cytotoxicity was determined by neutral red uptake.^eVCA ELISA.^fCytopathic effect (CPE) inhibition assay.^gCytotoxicity was determined in CEM cells.^h*E*-isomer.ⁱGanciclovir.^jAcyclovir.

by introduction of the PPA function. This was observed in VZV assays of pronucleotides **99b** through **99e** where the potentiating effect reached from 7.7 fold (**99b**) to 200 fold (**99d**). The *E*-isomer **100a** was effective in EBV assays with potentiating effect clearly apparent in

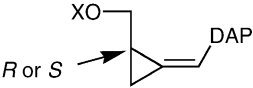
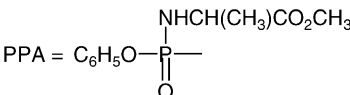
Daudi culture. The guanine PPA pronucleotide **100b** was effective only in EBV/H-1 culture with EC_{50}/CC_{50} 8/>50. The parent analogue **2b** had EC_{50} >50 μ M. By contrast, the 2,6-diaminopurine analogue **100c** exhibited activity in Daudi cells (EC_{50}/CC_{50} 4.0/>106), whereas compound **2f** was virtually inactive (EC_{50} 58 μ M).

The antiviral activity of more polar pronucleotide lacking the aromatic group (phenyl) **106a** (Figure 33) corresponded to that of the parent analogue **1a**.⁶⁹ Potentiation effects observed in the PPA analogue **99a** were absent. It is likely that the phosphamidate **106a** is hydrolyzed before entering the infected cell. A single exception was the *E*-isomer **106b** whose potency in EBV/Daudi (EC_{50}/CC_{50} 0.88/>92 μ M) surpassed the parent analogue **2a** (EC_{50}/CC_{50} 71/>230 μ M) and pronucleotide **100a** (EC_{50}/CC_{50} 3.2/65 μ M). It appears that in this particular instance, phosphamidate **106b** is capable of penetrating the cellular membrane. It was inactive or marginally effective (VZV/HFF) against other viruses.

The PPA pronucleotides discussed so far were derived from racemic methylenecyclopropanes, i.e., they are mixtures of four diastereoisomers each because the phosphorus atom in the PPA grouping is chiral. In one instance, pronucleotides “*R*”-**99c** and “*S*”-**99c** (two diastereoisomers each) related to 2,6-diaminopurine enantiomers **R-1f** and **S-1f** were investigated in detail.⁵³ Especially interesting are the different responses of enantiomers **R-1f** and **S-1f** to the potentiating effect of the PPA group. In the HCMV and MCMV assays the *S*-selectivity of the parent enantiomers was maintained (Table 16). In VZV/HFF culture, where the parent enantiomers had little potency, almost no differentiation between the “*R*”-**99c** and “*S*”-**99c** was observed, although the efficacy of both diastereoisomers was significantly increased. By contrast, no activity of both diastereoisomers against HSV-1 or HSV-2 was noted.

In EBV assays, the diastereoselectivity of “*R*”-**99c** and “*S*”-**99c** followed the enantioselectivity pattern of the parent methylenecyclopropanes (Table 17). Cytotoxicity of both diastereoisomers was also observed. The *R* and *S* series were almost equipotent in H-1 cells, whereas the only potentiation of anti-EBV effect was seen in VCA-FF assay for “*R*”-**99c**. The anti-HIV and anti-HBV activity relative to parent enantiomers were also increased for both diastereoisomers (Table 18), although the effects are weaker than with the corresponding “racemic” compounds **99c** (Table 13). Another pair of diastereoisomeric PPA analogues **8b** and **9b** derived from isoguanine methylenecyclopropanes **8a** and **9a** (Figure 2) was devoid of antiviral activity.⁴⁶

Table 16. Comparison of inhibition of human and murine cytomegalovirus (HCMV and MCMV) and varicella zoster virus (VZV) replication by diastereoisomeric 2,6-diaminopurine pronucleotides **“R”-99c** and **“S”-99c** with the parent enantiomers^a **R-1f** and **S-1f**

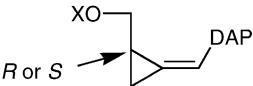
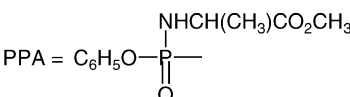
		DAP = 2,6-diaminopurine, X = H or PPA			
					
Compound	X	EC ₅₀ /CC ₅₀ (μM)			
		HCMV/HFF ^b		MCMV/MEF ^b	VZV/HFF ^{b,c}
		Towne	AD169		
“R”-99c	PPA	23/100	>42/211	>42/110	5.3
R-1f	H	>100/>100	>431/>431	>86/431	>431
“S”-99c	PPA	3/100	5.5/115	1.5/183	1.7
S-1f	H	21/>100	18/>431	0.65/431	68

^aData from Qiu et al.⁵³

^bPlaque reduction assay.

^cFor cytotoxicity see HCMV/AD169 assay.

Table 17. Comparison of inhibition of Epstein–Barr virus (EBV) replication by diastereoisomeric 2,6-diaminopurine pronucleotides **“R”-99c** and **“S”-99c** with the parent enantiomers^a **R-1f** and **S-1f**

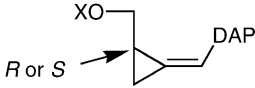
		DAP = 2,6-diaminopurine, X = H or PPA		
				
Compound	X	EC ₅₀ /CC ₅₀ (μM)		
		EBV/Daudi ^b		EBV/H-1 ^b
		VCA-IF	DNA hybridization	
“R”-99c	PPA	0.57/40	2.4	1.9 ^c
R-1f	H	12/>215	12	2.3
“S”-99c	PPA	2.5/16	toxic	2.8 ^c
S-1f	H	0.69/>215	60	1.3

^aData from Qiu et al.⁵³

^bFor assay descriptions see Table 3.

^cToxic at 23 μM.

Table 18. Comparison of inhibition of HIV-1 and HBV replication by diastereoisomeric 2,6-diaminopurine pronucleotides “**R**”-**99c** and “**S**”-**99c** with the parent enantiomers^a **R-1f** and **S-1f**

<div style="display: flex; align-items: center; justify-content: space-around;"> <div style="text-align: center;">  <p><i>R</i> or <i>S</i></p> </div> <div> <p>DAP = 2,6-diaminopurine, X = H or PPA</p> <p>PPA = $\text{C}_6\text{H}_5\text{O}-\text{P}(\text{NHCH}(\text{CH}_3)\text{CO}_2\text{CH}_3)_2$</p> </div> </div>			
Compound	X	EC ₅₀ /CC ₅₀ (μM)	
		HIV-1/CEM-SS ^{b,c}	HBV/2.2.15 ^{c,d}
“R”-99c	PPA	0.55/30	2.1
R-1f	H	>100/>100	10
“S”-99c	PPA	3.5/20	0.8
S-1f	H	>100/>100	5

^aData from Qiu et al.⁵³

^bReverse transcriptase assay.

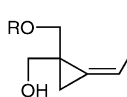
^cCytotoxicity was determined in CEM cells.

^dDNA hybridization assay.

The effects of the PPA function on the antiviral activity was also investigated in the second-generation series.⁷³ Again, the “rule” that antiviral activity of highly effective methylenecyclopropanes is rarely increased by attachment of the PPA moiety has found its application also in this group of analogues. Thus, in CMV assays, cyclopropavir (**4b**) was about 7–36 times more potent than pronucleotide **18b** (Table 19). By contrast, the adenine PPA prodrug **18a** was roughly equipotent with the parent compound **4a**.

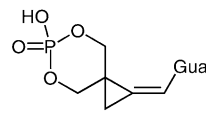
Not surprisingly, cyclopropavir phosphate (**14**) was as effective as cyclopropavir (**4b**) itself. Most likely, it is a more soluble prodrug of **4b** which undergoes dephosphorylation at the cellular membrane of infected cells as described for other nucleotides.⁶⁷ Of interest is the fact that phosphate **14** is as effective against HSV-1 and HSV-2 in HFF culture with EC₅₀ 9.9 and 23.3 μM, although cyclopropavir (**4b**) and PPA analogue **18b** are inactive. This may indicate that intact analogue **14** is capable of penetrating, at least to some extent, the cellular membrane. The cyclic phosphate **16** is perhaps the most interesting compound of this group of analogues. It is effective against the AD169 strain of HCMV and MCMV and somewhat less against the Towne strain. The anti-HCMV effect in AD169 is comparable with ganciclovir cyclic phosphate (**31**).^{15,63}

Table 19. Inhibition of HCMV and MCMV replication by phosphate derivatives of the second-generation methylenecyclopropane analogues^a



B = Ade or Gua
R = H, PPA or PO₃H₂

PPA = C₆H₅O-P(=O)(NHCH(CH₃)CO₂CH₃)-



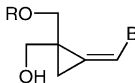
16

Compound	B	R	EC ₅₀ /CC ₅₀ (μM)		
			HCMV/HFF ^b		MCMV/MEF ^b
			Towne ^c	AD169 ^d	
18a	Ade	PPA	3/32	18.4/200	not tested
4a	Ade	H	2.4/>100	11.7/>404	9.7/>404
18b	Gua	PPA	3.6/>100	8.1/>198	25.2/>198
4b	Gua	H	0.33/100–200	0.49/>380	0.27/>380
14	Gua	PO ₃ H ₂	0.25/100	1.1/>291	0.26/175
16			20/>100	6.0/>301	7.2/>301
Ganciclovir			2.1/>100	1.6/>392	2.1/>392

^aData from Yan et al.⁷³^bPlaque reduction assay.^cVisual cytotoxicity.^dCytotoxicity by neutral red uptake.


Although the CMV results may be compatible with a minute hydrolysis of **16** to phosphate **14** or cyclopropavir (**4b**), the EBV (Daudi) and HBV assays seem to rule this out (Table 20).⁷³ Thus, cyclic phosphate **16** is the most potent and non-cytotoxic analogue in both assays, whereas **14** and **4b** are inactive. Compound **16** then resembles ganciclovir cyclic phosphate (**31**) that has an intrinsic antiviral activity not associated with conversion to ganciclovir.^{17,43} Cyclic phosphate **16** was inactive in EBV/H-1 assay. By contrast, the PPA pronucleotides **18a** and **18b** were effective against EBV in H-1 cells with some cytotoxicity but they lacked any significant potency in Daudi cells. Phosphate **14** was also active in H-1 cells acting as a prodrug of cyclopropavir (**4b**). The only analogue effective against VZV/HFF (plaque reduction) with EC₅₀/CC₅₀ (μM) 7.3/>198 was the guanine PPA derivative **18b** which was >27 times more potent than cyclopropavir (**4b**) and non-cytotoxic. The *E*-isomers **15**, **17**, **19a** and **19b** were devoid of antiviral activity.

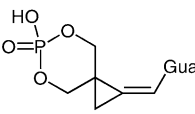
Table 20. Inhibition of EBV and HBV replication by phosphate derivatives of the second-generation methylenecyclopropane analogues^a



B = Ade or Gua
R = H, PPA or PO₃H₂

PPA = C₆H₅O—





16

Compound	B	R	EC ₅₀ /CC ₅₀ (μM)		
			EBV		HBV
			Daudi ^b	H-1 ^{c,d}	2.2.15 ^{d,e}
18a	Ade	PPA	>102/>102	3/3.8	12.8
4a	Ade	H	166/>202	>20/>50	>10
18b	Gua	PPA	>99/>198	3.4/35	4.1
4b	Gua	H	45/74	7/>50	>10
14	Gua	PO ₃ H ₂	>146/>146	2.3/62	15
16			0.96/>150	>20/>100	0.8
Control			1.1/>222 ^f	5 ^g	0.05/>100 ^h

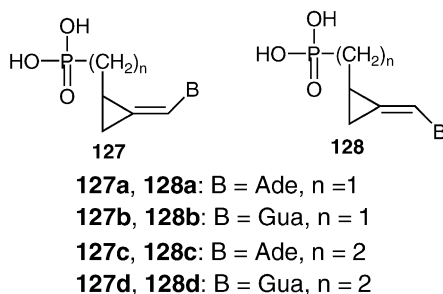
^aData from Yan et al.⁷³^bViral capsid immunofluorescence (VCA) ELISA.^cDNA hybridization assay.^dCytotoxicity was determined in CEM cells.^eFor CC₅₀ values see EBV/H-1 assays.^fAcyclovir.^gGanciclovir.^hLamivudine.

E. Phosphonates of methylenecyclopropane analogues

Phosphonates of the first-generation methylenecyclopropane analogues **127a–127d** and **128a–128d** did not exhibit significant antiviral activity (Figure 40).²⁰ One of the reasons may be their inability to penetrate the cellular membrane owing to a lack of substrate activity for specific transporters.¹² From a new set of phosphonates **20a**, **20b**, **21a** and **21b** (Figure 2) designed by a different rationale (see Section II), the *E*-isomer **21b** was highly effective against EBV/Daudi with EC₅₀/CC₅₀ <0.03/>300 μM surpassing *Z*-isomer **20b** (EC₅₀/CC₅₀ 1.1/>300 μM) but it was inactive against other viruses.⁷⁴

F. Mechanism of action of methylenecyclopropane analogues

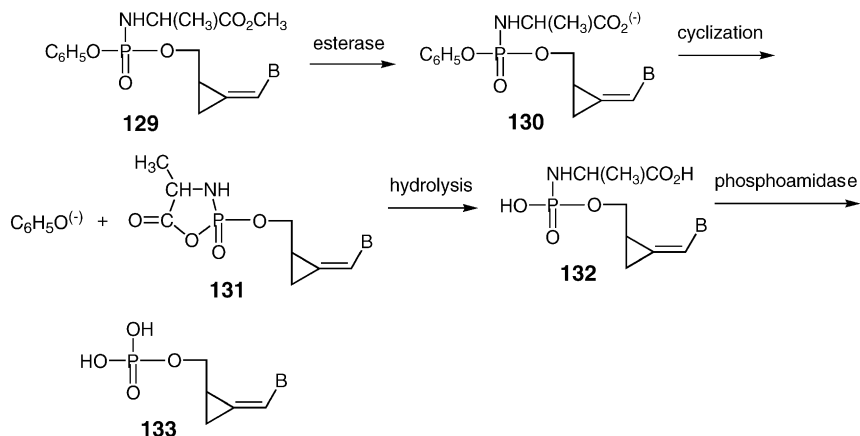
Since the beginning, it has been anticipated that methylenecyclopropane analogues undergo the usual sequence established for nucleo-

*Figure 40.*

sides and their analogues, methylenecyclopropane \rightarrow monophosphate \rightarrow diphosphate \rightarrow triphosphate \rightarrow DNA. Comparison of synadenol (**1a**) and synguanol (**1b**) with ganciclovir has established that inhibition of HCMV DNA synthesis by the respective triphosphates is a likely mode of action of these analogues. This was also supported by time of addition studies^{14,80} showing that synguanol (**1b**) acted late in the viral replication cycle.

As already mentioned, the first indication that monophosphates are involved in the first step of the activation came from studies of antiviral activity of the phenyl phosphoralaninate (PPA) pronucleotides (Section D). It has been established^{8,77} that conversion of PPA derivatives of nucleoside analogues to monophosphates proceeds as follows (Figure 41): two crucial enzyme types are involved, intracellular esterases and phosphoamidase.⁵⁸ A similar mode of action is anticipated for PPA methylenecyclopropanes. In the first step, the alanine ester group of **129** is cleaved by esterase to give intermediate **130** which is, in turn, cyclized to cyclic anhydride **131** with the expulsion of the phenoxy group. A non-enzymic hydrolysis leads then to phosphoamidic acid **132**. The phosphoamidase converts **132** to monophosphate **133**. All PPA methylenecyclopropane analogues that have been tested for antiviral activity, active and inactive, are substrates for porcine liver esterase (PLE) used as a model for intracellular esterases. These phosphoramidates include **8b**, **9b** (Figure 30),⁴⁶ **99a–99e**, **100a** (Figure 28),⁴⁷ **12a**, **12b**, **13a**, **13b**, **104a** and **104b** (*Z*- and *E*-isomers, Figures 31, 32)¹ and **18a**, **18b**, **19a**, **19b** (Figure 35 and Chart 1).⁷³ Nevertheless, activity of products of esterase cleavage **132** for phosphoamidase has not yet been investigated.

The final proof that triphosphates are the species responsible for antiviral activity of methylenecyclopropane analogues came from the inhibition of HIV-1 reverse transcriptase by the *Z*- and *E*-triphosphates

*Figure 41.*

of synadenol **105a** and **105b** (Figure 33).⁷¹ The *Z*-triphosphate **105a** was as effective as ddATP.

The first phosphorylation step is usually considered as the most important for antiviral activity of nucleoside analogues. Several lines of evidence implicated UL97-encoded phosphotransferase of human cytomegalovirus as the phosphorylation agent for synadenol (**1a**) and synguanol (**1b**)² and cyclopropavir (**4b**).²⁸ This enzyme is also responsible for phosphorylation of ganciclovir.⁶² The results with ganciclovir (**30**), synadenol (**1a**) and synguanol (**1b**) indicated that HCMV mutant with a large deletion of UL97 sequence is about equally resistant to all three analogues. By contrast, both **1a** and **1b** were fully potent against a number of HCMV clinical isolates resistant to ganciclovir and containing only a single point mutation.² Similar results were obtained with cyclopropavir (**4b**) although the resistance to a mutant with a large deletion of UL97 was about half of that observed with ganciclovir (**30**).²⁸ Taken together, it is likely that phosphorylation of methylenecyclopropanes **1a**, **1b** and **4b** is catalyzed by UL97 phosphotransferase, although a direct evidence is still missing. Nevertheless, experiments with single point mutations of UL97 indicate significant differences of substrate activity of **1a**, **2a** and **4b** relative to ganciclovir.

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SYNTHESIS AND ANTIVIRAL EVALUATION OF BROAD SPECTRUM, ORALLY ACTIVE ANALOGS OF CIDOFOVIR AND OTHER ACYCLIC NUCLEOSIDE PHOSPHONATES

Karl Y. Hostetler

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I. INTRODUCTION

Acyclic nucleoside phosphonates are an important class of antiviral agents first reported and evaluated by the groups of De Clercq and Holy.^{11,25} These compounds are nucleoside bases having an acyclic moiety terminated by a phosphonate residue instead of a ribose or deoxyribose. Normal nucleosides are activated by three anabolic phosphorylations to form their triphosphates. The phosphonate residue eliminates the need for the first phosphorylation which is often rate limiting in the conversion of the nucleoside antiviral analog to its triphosphate.

Three acyclic nucleoside phosphonates, (S)-9-(3-hydroxy-2-phosphonomethoxy)cytosine (HPMPC, cidofovir), 9-(2-phosphono-methoxyethyl)adenine (PMEA, adefovir) and 9-[2-(R)-(phosphonomethoxy)propyl]adenine (PMPA, tenofovir), are currently marketed for treatment of HCMV, HBV and HIV, respectively.¹⁰ Compounds of this class have several disadvantages. Because of the two negative charges, acyclic nucleoside phosphonates enter cells slowly by fluid phase endocytosis⁸ and have low oral bioavailability⁹ unless they are masked by various types of protecting groups. Cidofovir (Vistide®) is not orally

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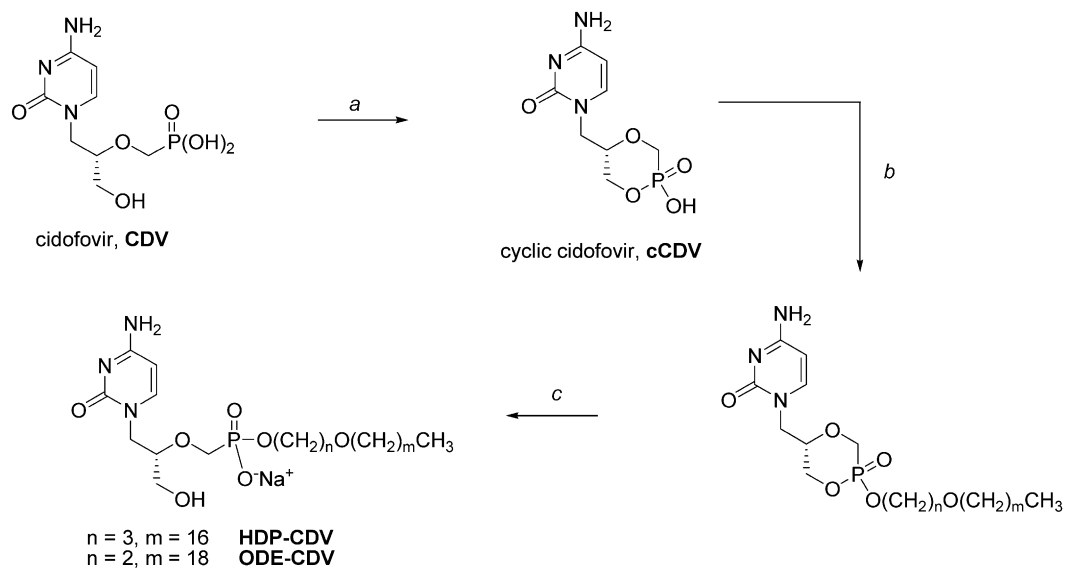
bioavailable and is given intravenously. However, adefovir dipivoxil (Hepsera®) and tenofovir disoproxil (Viread®) are marketed drugs which have charge masking groups which are cleaved during absorption from the small intestine. Adefovir and tenofovir are released and circulate in the plasma. The dose limiting toxicity of acyclic nucleoside phosphonates is nephrotoxicity, a disadvantage due to their rapid uptake by an organic anion transporter and concentration in the kidney proximal tubule.⁹

Over the past few years, our laboratory has developed an alternative approach to the problems posed by the antiviral nucleoside phosphonates. We have employed a chemical modification which disguises acyclic nucleoside phosphates as analogs of lysophosphatidylcholine (LPC). LPC is formed in the gut by the action of pancreatic phospholipase A₂ on dietary phosphatidylcholine (lecithin). A significant proportion of LPC has been shown to be absorbed intact from the small intestine.^{5,24} Our approach involves esterification of one of the phosphonate—OH groups with various lipid alkoxyalkyl or alkyl esters so that the conjugate resembles LPC. The compounds are generally stable in plasma. After uptake in tissues, cellular enzymes metabolize the conjugate to the nucleoside phosphonate which is phosphorylated twice by cellular enzymes to the active metabolite. This is a strategy which is applicable to cidofovir and also to most other antiviral phosphonates. This chapter will concentrate on cidofovir as the prototype for alkoxyalkyl and alkyl esters of acyclic nucleoside phosphonates.

II. SYNTHESIS OF HEXADECYLOXYPROPYL AND RELATED ESTERS OF CIDOFIVIR

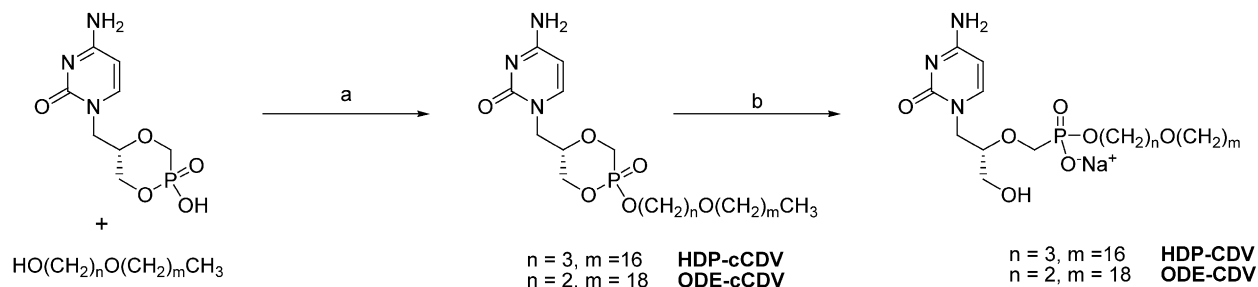
Esters of cidofovir were synthesized either by linking the alkoxyalkyl-bromides ([Method 1](#)) or the alkoxyalkanols ([Method 2](#)) to cyclic cidofovir, followed by opening of the ring with base. Alkyl esters may be made by either method.

METHOD 1. (See [Scheme 1](#).) The appropriate 1-bromoalkoxyalkane (or 1-bromoalkane) was added to a suspension of cyclic CDV dihydrate, N,N-dicyclohexyl-4-morpholino-carboxamidine and N,N-dimethylformamide and the mixture was allowed to react at 60 °C overnight. The solvent was evaporated under reduced pressure and the residue was purified by flash column chromatography on silica gel with an elution gradient of dichloromethane to 15% ethanol. The various cyclic CDV esters were isolated as equimolar mixtures of the axial and equatorial diastereomers in yields of 30 to 40%.^{2,27}



Reagents: (a) N,N-dicyclohexyl-4-morpholinocarboxamidate, DCC, pyridine, 100 °C; (b) 1-alkoxy-3-bromoalkanes, N,N-DMF 80 °C; (c) 0.5 M NaOH.

Scheme 1. Alkylation of cyclic CDV with 1-bromo-3-alkoxyalkanes.



Reagents: (a) triphenylphosphine, diisopropylazodicarboxylate, N,N-DMF; (b) 0.5 N NaOH.

Scheme 2. Alkoxyalkanol coupling to cyclic CDV.

METHOD 2. (See [Scheme 2](#).) Alkoxyalkyl analogs may also be synthesized using an alternative procedure employing the condensation of the appropriate alkoxyalkanol with cyclic CDV.²⁷ Anhydrous cCDV, the appropriate alkoxyalkanol and triphenylphosphine were dissolved/suspended in anhydrous N,N-DMF, and stirred vigorously under nitrogen. Diisopropyl azadicarboxylate was then added and the mixture was allowed to react overnight. The solvent was then evaporated under vacuum and the residue was adsorbed onto silica gel and purified by silica gel column chromatography with a gradient elution of 100% dichloromethane to 15% ethanol followed by recrystallization from *p*-dioxane. The coupled products were isolated as equimolar mixtures of axial and equatorial diastereomers. This method was also used to make the alkyl esters by coupling the indicated alcohol. Yields generally ranged from 22 to 45%. [Method 2](#) was found to be more suitable for the synthesis of large quantities of these compounds.

RING OPENING. The alkyl- or alkoxyalkyl-cyclic CDV analogs were converted to the open form by exposure to 2M NaOH at 80 °C for one hour, during which time the mixtures became clear. The solutions were cooled to room temperature and acidified with glacial acetic acid to pH 5. The resulting precipitates were collected by vacuum filtration, dried and purified either by flash column chromatography with silica gel or recrystallized to purity from ethanol.²⁷ Yields ranged from 32 to 85%.

III. ANTIVIRAL EVALUATION AND SPECTRUM OF ACTIVITY

We tested various alkoxyalkyl and alkyl esters in cells infected with poxviruses, herpesviruses, adenoviruses and a polyoma virus ([Table 1](#)). Esterification of CDV with HDP-, ODE- or eicosyl-(EC) esters increased the antiviral activity in every case against all viruses tested. Against the poxviruses, vaccinia Copenhagen, cowpox Brighton, ectromelia, variola Bangladesh, the EC₅₀ of CDV ranged from 12 to 31 μM compared with 0.1–0.6 μM for HDP-CDV, 0.03–0.23 μM for ODE-CDV and 1.5–1.6 μM for EC-CDV, respectively, representing increases of activity versus CDV of 27 to 900 fold. Against poxviruses, the order of antiviral activity was ODE-CDV > HDP-CDV > EC-CDV >> CDV. Variola Bangladesh appeared to be the poxvirus most sensitive to the esters of CDV in vitro; ODE-CDV was the most active analog against variola with an EC₅₀ of 0.03 μM, a 900 fold increase compared with

Table 1. Antiviral activity and selectivity of CDV and its esters in cells infected with various DNA viruses

Virus	EC ₅₀ , μM				S.I. range	Ref.
	CDV	HDP-CDV	ODE-CDV	EC-CDV		
POXVIRUSES						
Vaccinia CS	31	0.6	0.2	1.6	30–105	27,17,16
Cowpox BR	42	0.5	0.2	1.5	28–105	27,17,16
Ectromelia	12	0.45	0.23	–	–	6
Variola BD	27	0.10	0.03	–	–	15
HERPESVIRUSES						
HCMV	1.2	0.0009	0.0009	0.70	5,500–93,000	2,27
MCMV	0.04	0.0009	0.001	0.005	7,700–84,000	2,27
HSV-1	5.5	0.06	0.02	0.1	–	28
HSV-2	5.1	0.08	0.03	0.3	–	28
HHV-6A	2.7	0.004	0.003	0.3	–	28
HHV-6B	5.4	0.007	<0.003	0.6	–	28
HHV-8	2.6	0.02	0.030	0.05	–	28
VZV	0.5	0.0004	0.0001	0.01	–	28
EBV (DNA red)	65.6	0.03	0.10	–	–	28
ADENOVIRUSES						
AdV 3	2.0	0.01	<0.06	0.04	>158–>4300	13
AdV 5	0.5	<0.009	<0.008	0.01	>634–>4300	13
AdV 7	1.3	0.02	<0.009	0.07	>243–1950	13
AdV 8	2.0	0.03	<0.008	0.025	>342–1540	13
AdV 31	>48	0.28	0.09	0.05	>226–770	13
POLYOMA VIRUS						
BK virus	115	0.13	0.083	–	104–20	22

Data are expressed as μ M EC₅₀ values and are the average of 2 to 5 determinations. A dash indicates not tested. Abbreviations: vaccinia CA, vaccinia Copenhagen, cowpox BR, cowpox Brighton; variola BD, variola Bangladesh; HCMV, human cytomegalovirus; AdV, adenovirus types 3,5,7,8 and 31. S.I., selectivity index, CC₅₀/EC₅₀; S.I. range refers to the selectivity index range of the HDP-, ODE- and EC-CDV derivatives.

CDV. The CDV esters were also highly active against all herpesviruses tested. The greatest antiviral activity was apparent against murine and human cytomegalovirus and varicella-zoster virus with EC₅₀ values in the 1 nanomolar range representing an increase of 1,300 fold (HCMV), 40 fold (MCMV) or 1,200 to 5,000 fold (VZV). Marked increases in activity versus CDV were also noted for HSV-1, HSV-2, EBV

and the various HHV viruses. Notably, HDP-CDV and ODE-CDV had EC_{50} values in the 3 to 30 nanomolar range against HHV-6a, HHV-6b and HHV-8.²⁸ EC-CDV, an alkyl ester of CDV, showed increased antiviral activity against herpesviruses, but was generally less active than the alkoxyalkyl series. The esters of CDV were also substantially more active than CDV against a polyoma virus and 5 strains of adenovirus (Table 1).^{13,22}

IV. STRUCTURE ACTIVITY RELATIONSHIPS

To examine the effect of the lipid structure on the antiviral activity and selectivity of the cidofovir analogs, we synthesized a family of alkoxypropyl-CDVs varying in overall chain length from 8 to 24 atoms. We also synthesized a series of alkyl-CDVs lacking the propanediol linker moiety. Their antiviral effects were examined against HCMV, *in vitro* (Figure 1). Compounds having chains less than 18 carbon atoms showed a progressive loss of antiviral activity as the chain was further shortened. Compounds having 22 atoms were also less active, but as the chain was lengthened further to 24 atoms, antiviral activity decreased (Figure 1). In MRC-5 cells infected with HCMV the optimal length of either the alkyl or alkoxyalkyl ester moiety was observed to be 20 atoms.²⁷

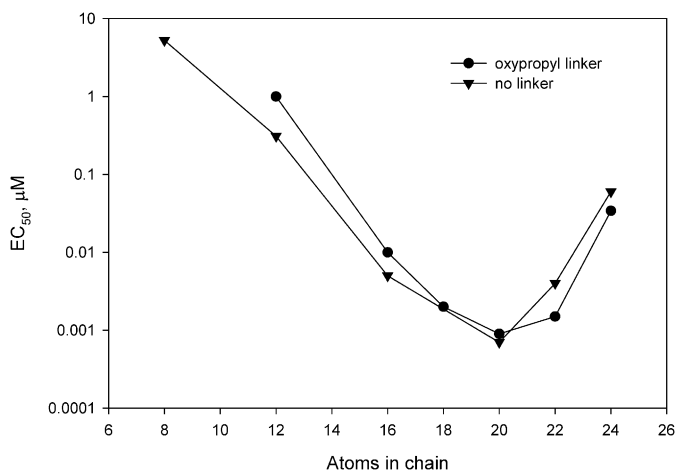
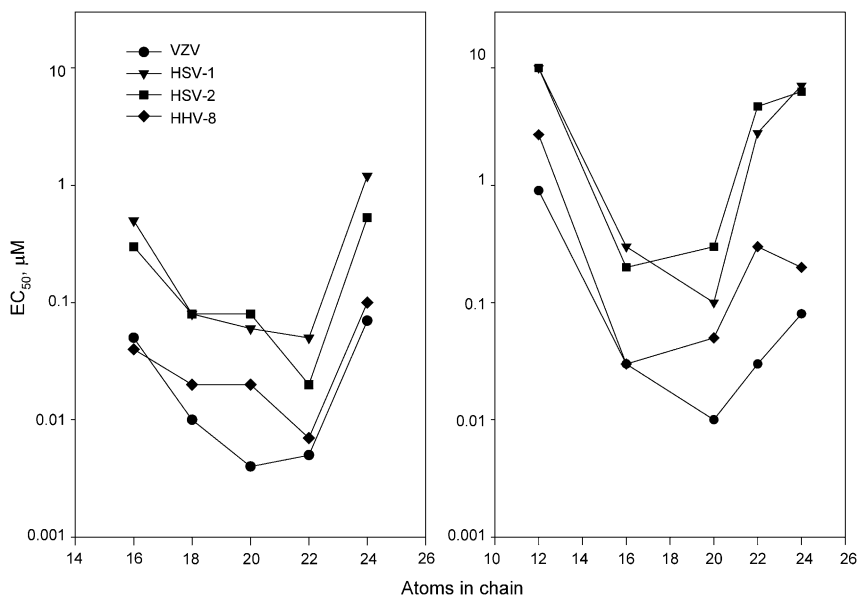


Figure 1. Effect of alkoxyalkyl and alkyl chain length on the antiviral activity of CDV esters against human CMV *in vitro*.



Adapted from Williams-Aziz et al.²⁸

Figure 2. Effect of alkoxyalkyl (left panel) and alkyl (right panel) chain length on the antiviral activity of CDV esters against HSV-1, HSV-1, HHV-8 and VZV, in vitro.

We also tested the series of analogs of CDV against other viruses, including HSV-1, HSV-2, HHV-8 and VZV (Figure 2). The alkyl-CDV series showed optimal activity against HSV-1 and VZV at 20 atoms while HSV-2 and HHV-8 had optimal chain lengths of 18 atoms. In general, the alkyl esters of CDV (right panel) were less active than the alkoxyalkyl-CDV esters (left panel). The alkoxyalkyl-CDV series showed the greatest antiviral activity at a chain length of 22 atoms against HSV-1, HSV-1 and HHV-8, while antiviral activity against VZV was optimal at 20 atoms. The same trends were noted against vaccinia and cowpox viruses¹⁶ and EBV.²⁸

The reasons for the dependence of antiviral activity on the length of the lipid chain are not entirely clear. We speculate that the short chains are more water soluble and lack the ability to associate with the plasma membrane of the infected cell, reducing cellular uptake. Less active compounds of this class having alkoxyalkyl chains of 24 atoms may be less fluid, resulting in slower metabolism, delaying or reducing the release of intracellular cidofovir.

V. CELLULAR METABOLISM

As noted above, CDV analogs, HDP-CDV, ODE-CDV and EC-CDV, show >100-fold increases in antiviral activity versus unmodified CDV against cells infected with various DNA viruses including orthopoxviruses, herpesviruses and adenoviruses (Table 1). To assess the metabolic basis for the increased antiviral activity of HDP-CDV *in vitro*, we studied the cellular uptake and metabolism of ^{14}C -labeled CDV and HDP-CDV in MRC-5 human lung fibroblasts. MRC-5 cells were exposed to 10 μM CDV or HDP-CDV for times ranging from 1 to 24 hours and drug uptake was assessed. Cellular uptake of CDV was maximal at 1 to 4 hours and did not increase further over the next 20 hours. However, cell associated HDP-CDV increased nearly linearly for 6 hr and progressively to 24 hrs.¹ The metabolites of HDP-CDV were evaluated by HPLC and the amount of cellular CDV, CDV monophosphate and CDV diphosphate were determined (Table 2). At 24 and 48 hours, the amount of CDV diphosphate formed from HDP-CDV was 132 and 184 picomoles per flask versus 1.3 and 1.8 picomoles/flask with CDV, representing an increase of 100 fold.¹ Wash out experiments showed that in cells exposed to HDP-CDV, CDV diphosphate had a half life of about 8 days. Thus, the increased uptake and conversion of HDP-CDV to CDV diphosphate in MRC-5 cells appears to explain the marked increase observed in antiviral activity against poxviruses and cytomegalovirus.

Figure 3 shows a proposed mechanism to explain the increased cell uptake and metabolism to CDV diphosphate observed with HDP-CDV and related compounds. CDV itself is taken up by fluid endocytosis, a slow process,⁸ but HDP-CDV can insert into the plasma membrane phospholipid bilayer and may enter the cell quickly by flip-flop followed by desorption from the inner leaflet of the plasma membrane. This is due in part to its single alkyl chain since phospholipids having the normal two chain structure are known to have very slow rates of flip-flop.^{20,23} Alternatively, flip-flop of HDP-CDV and related compounds may be protein mediated.¹⁹ Once in the cell, HDP-CDV is converted to hexadecylpropanediol and CDV by a cellular phospholipase C or other phosphatase. In some tissues, e.g. liver, HDP-CDV may undergo oxidative metabolism. The CDV liberated by metabolism is phosphorylated twice by cellular enzymes to CDV-diphosphate, the active antiviral. Much greater levels of CDV diphosphate result from exposure of cells to HDP-CDV than CDV as we have shown in MRC-5 cells *in vitro* (Table 2).

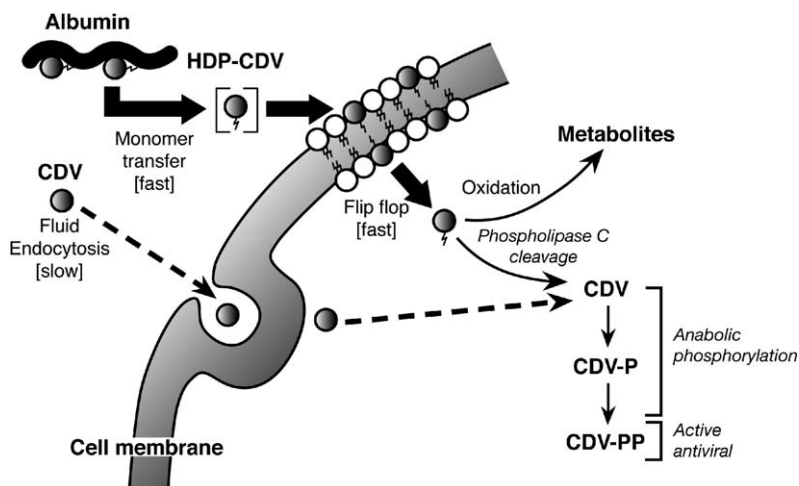


Figure 3. Proposed mechanism of cellular uptake and activation of HDP-cidofovir versus cidofovir.

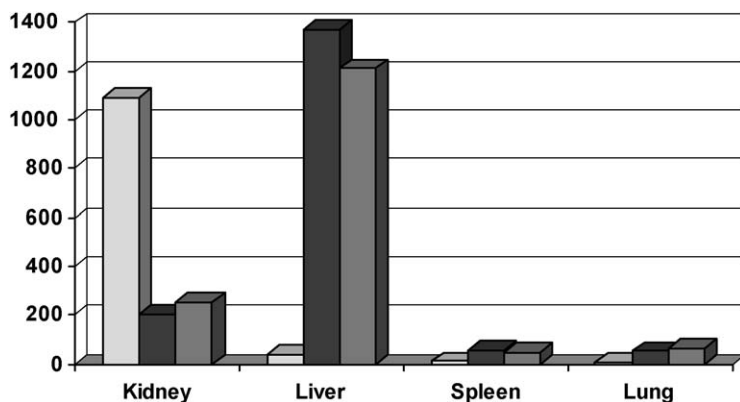
Table 2. Metabolite levels in MRC-5 cells following exposure to [2-¹⁴C]-CDV or HDP-[2-¹⁴C]-CDV

Metabolite	CDV		HDP-CDV	
	24 hr	48 hr	24 hr	48 hr
CDV	274	129	697	702
CDV-monophosphate	1.0	1.2	63	71
CDV-diphosphate	1.3	1.8	132	184

Data in picomoles/flask. Indicated times are the time of exposure to radioactive 10 μ M CDV or HDP-CDV. Each data point represents an analysis of the cells from a single T-75 flask. Abbreviations: CDV, cidofovir; Adapted from Aldern et al.¹

VI. ORAL PHARMACOKINETICS AND TISSUE DISTRIBUTION

Experiments to assess the oral bioavailability of HDP-CDV and ODE-CDV in mice were carried out to see if the conjugation of an alkoxyalkanol to the phosphonate of CDV results in efficient uptake from the small intestine.⁷ [2-¹⁴C]-CDV, HDP-[2-¹⁴C]-CDV and ODE-[2-¹⁴C]-CDV (10 mg/kg) were administered to mice by the intraperitoneal or oral routes and plasma and tissues were obtained at times ranging from 1 to 72 hr. The level of HDP-CDV and metabolites in



Intraperitoneal CDV, left bar; oral HDP-CDV, center bar; oral ODE-CDV, right bar. AUC as nanomol.hrs/gm from 0 to 72 hrs. Adapted from Ciesla et al.⁷

Figure 4. Tissue area under curve: Intraperitoneal CDV versus oral HDP-CDV or ODE-CDV in mice.

plasma following the oral dose peaked at 3 hr at 2.37 μM . Analysis of the plasma by lipid extraction and chromatography of the extraction indicated that about 50% of the radioactivity was intact HDP-CDV, the remainder being CDV and metabolites.⁷

Comparison of the oral and parenteral areas under curve (AUC) indicated that the relative oral bioavailability of HDP-CDV and ODE-CDV was estimated to be 88% and 93%, respectively.⁷ For comparison, the oral bioavailability of CDV in rats has been reported to be <3%. HDP-CDV persists in plasma following absorption in mice and is converted to CDV and metabolites in the tissues. Plasma CDV is thought to result from tissue metabolism and release into plasma because HDP-CDV is stable in plasma for several days at 37 °C.

The tissue distribution of CDV, HDP-CDV and ODE-CDV and metabolites was determined using ^{14}C -labeled drugs and the C_{max} and area under curve (AUC) of drug and metabolites was plotted. The C_{max} for intraperitoneal CDV in kidney was 180 nanomoles/gm, 30 times that observed for oral HDP-CDV or ODE-CDV.⁷ The area under curve analysis is shown in Figure 4. Several notable trends were apparent. When CDV is given intraperitoneally, most of the radioactive CDV appeared in the kidney and very little CDV was noted in liver, spleen and lung. In contrast, oral HDP-CDV and ODE-CDV delivered large amounts of the drug and metabolites to liver while the AUC of HDP-CDV and ODE-CDV in kidney was only 19 to 24% of that observed with intraperitoneal CDV. CDV is avidly taken up by organic anion

transporters in kidney, but the lipid esters do not appear to concentrate in kidney. With oral HDP-CDV and ODE-CDV administration, it is possible that much of the AUC in kidney is accounted for by tissue metabolism of these drugs to CDV which is subsequently taken up by the kidney organic anion transporter.

It should be noted that the alkoxyalkyl ester strategy differs from most prodrug approaches to improve oral absorption. For example, the prodrug, valacyclovir (Valtrex®), is converted to acyclovir during absorption and acyclovir circulates in plasma. Similarly, tenofovir disoproxil fumarate (Viread®) and adefovir dipivoxil (Hepsera®) are converted to tenofovir and adefovir which circulate in plasma. In contrast, alkoxyalkyl nucleoside phosphonates, like HDP-CDV, persist after absorption and circulate intact in plasma; after uptake in tissues they are metabolized to their diphosphates, the active antiviral moiety (see Figure 3).

VII. ANTIVIRAL ACTIVITY IN ANIMAL MODELS OF VIRAL DISEASES

A. Poxvirus infections

Ectromelia infection in mice

The effect of oral treatment with CDV, HDP-CDV and ODE-CDV was evaluated in mice given a lethal aerosol challenge with ectromelia virus. Drugs were administered orally daily for 5 days beginning four hours following infection and mortality was assessed at 21 days. At day 7, several animals were sacrificed and tissues obtained for assessment of viral titers.⁶ Both HDP-CDV and ODE-CDV prevented death from a lethal challenge with ectromelia virus (Table 3). The viral titers

Table 3. Oral treatment of ectromelia infection with HDP-CDV and ODE-CDV

Treatment	% Mortality	MDD	Tissue viral titers		
			Spleen	Liver	Lung
Vehicle control	100	6.3	5.0×10^6	9.6×10^6	3.0×10^6
CDV 10 mg/kg \times 5	100	9.8	3×10^6	3×10^6	3.8×10^6
HDP-CDV 10 mg/kg \times 5	0	NA	$<1 \times 10^2$	$<1 \times 10^2$	5.0×10^4
ODE-CDV 10 mg/kg \times 5	0	NA	$<1 \times 10^2$	$<1 \times 10^2$	5.0×10^4

Infection with Ectromelia virus, 2.3×10^4 pfu by small particle aerosol, A/NCR mice, tissue titers on day 7 in pfu/ml; MDD, mean day of death; % mortality on day 21 Adapted from Buller et al.⁶

in liver and spleen on day 7 were below the limit of detection, while lung titers were reduced 1 to 2 logs.

Vaccinia and cowpox infection

In studies with intranasal infection of mice with vaccinia virus,²¹ HDP-CDV and ODE-CDV were given orally for 5 days at a dose of 5 mg/kg day. When treatment was delayed 24 hrs after infection HDP-CDV and ODE-CDV were effective in preventing death from disease with a mortality of 13% or nil, respectively (Table 4). Further delaying treatment for 48 or 72 hrs, resulted in increased mortality of 40–67% (HDP-CDV) and 93–100% (ODE-CDV), respectively.

In single dose experiments, mice were treated with a larger single dose of either CDV (intraperitoneal) or with oral HDP-CDV or ODE-CDV up to 5 days prior or 3 days after intranasal infection with cowpox virus²¹ (Table 5). A single dose of HDP-CDV of 12.5 mg/kg given 3 or 1 days before infection or 1 day after infection provided full protection. Cidofovir given intraperitoneally at day –1 or +1 also provided full protection. A single dose of 10 mg/kg of ODE-CDV was also effective from –5 to +3 days following infection with only 1 or 2 deaths in a group of 15 animals. This emphasizes the long intracellular half life of CDV-diphosphate, the active metabolite discussed previously. A single oral dose of HDP-CDV or ODE-CDV may provide significant antiviral activity in vivo for almost a week following administration. Thus, it appears that alkoxyalkyl esters of CDV may be useful for the prevention or treatment of poxvirus infection.

Table 4. Oral treatment of vaccinia infection in mice: effect of treatment delay

Treatment	Treatment delay, hrs	% Mortality
Placebo	24	100
HDP-CDV		
5 mg/kg	24	13
5 mg/kg	48	67
5 mg/kg	72	93
ODE-CDV		
5 mg/kg	24	0
5 mg/kg	48	40
5 mg/kg	72	100

Animals were infected with intranasally with vaccinia (15/group) and treated by gavage daily for 5 days with water, HDP-CDV or ODE-CDV as indicated, 24, 48 or 72 hours after infection. Adapted from Quenelle et al.²¹

Table 5. Oral treatment of cowpox infection in mice: effect of a single oral dose given prior to or following infection

Treatment	% Mortality				
	−5 days	−3 days	−1 day	+1 day	+3 days
Placebo	−	−	−	60%	−
CDV 30 mg/kg ip	27%	40%	0	0	7%
HDP-CDV 12.5 mg/kg	20%	0	0	0	13%
ODE-CDV 10 mg/kg	7%	0	14%	7%	7%

Animals (15/group) were infected intranasally with cowpox Brighton at indicated times before or after a single oral of CDV, HDP-CDV or ODE-CDV. Adapted from Quenelle et al.²¹

B. Cytomegalovirus infections

Human cytomegalovirus infection

The effect of oral HDP-CDV and ODE-CDV on human cytomegalovirus (HCMV) was evaluated in SCID-hu mice bearing infected thy/liv implants.⁴ Biopsies were obtained and HCMV titers determined (Table 6). Mice treated with vehicle had average HCMV log titers of 5.70 versus 3.30 for CDV treated animals. HDP-CDV (10 and 5 mg/kg) and ODE-CDV (5 mg/kg) treated animals did not have detectable HCMV in the Thy/liv implants at day 28. Generally similar results were obtained when SCID-hu mice with human fetal retinal implants were used. On a molar basis HDP-CDV and ODE-CDV given orally were estimated to be 4 to 8 times more efficacious against HCMV than intraperitoneal CDV.⁴

Table 6. Effect of oral HDP-CDV and ODE-CDV on in SCID-hu mice with Thy/Liv implants infected with HCMV in vivo

Treatment	HCMV pfu/gm on day 28	% positive for HCMV on day 28
Vehicle	5.70	82%
CDV, 20 mg/kg	3.30	20%
HDP-CDV, 10 mg/kg	0	0
HDP-CDV, 5 mg/kg	0	0
ODE-CDV, 5 mg/kg	0	0
ODE-CDV, 2.5 mg/kg	2.61	27

Data are pfu/gm log₁₀ and are the average of 10 or 11 determinations. Treatment was started 24 hrs following infection. Treatment was given daily for 28 days and was oral except for CDV, which was given intraperitoneally. Adapted from Bidanset et al.⁴

Murine cytomegalovirus infection

In lethal murine CMV infection, HDP-CDV and ODE-CDV were highly active in vitro against MCMV, guinea pig CMV and rat CMV with EC_{50} values of 1 to 9 nanomolar. When given orally at doses of 2.0 and 6.7 mg/kg daily for five days, HDP-CDV and ODE-CDV were effective when given 24 or 48 hours post infection. Statistically significant efficacy was also noted with a single oral dose or twice weekly doses of HDP-CDV or ODE-CDV versus placebo.¹⁸ In animals treated daily with HDP-CDV or ODE-CDV, virus titers in lung, liver, spleen, kidney, pancreas, salivary gland, and blood were reduced 3 to 5 logs which was comparable to CDV given i.p. These results indicated that HDP-CDV or ODE-CDV given orally was as effective as parenteral CDV for the treatment of experimental MCMV infection.¹⁸

VIII. ALKOXYALKYL ESTERS OF (S)-HPMPA AND OTHER ACYCLIC NUCLEOSIDE PHOSPHONATES

Most other acyclic nucleoside phosphonates have disadvantages similar to those of CDV, namely, poor oral absorption, slow membrane penetration and dose limiting nephrotoxicity. To see if the alkoxyalkyl esterification strategy would improve the antiviral activity and selectivity of other acyclic nucleoside phosphonates, we prepared the hexadecyloxypropyl esters of HPMPA, PMEA, PMEDAP, PME-N⁶-cyclopropyl-DAP and PME-5FC and evaluated their antiviral activity against cells infected with HIV-1.

The hexadecyloxypropyl ester of each acyclic nucleoside phosphonate was substantially more active against HIV-1 than their respective unmodified precursors (Table 7). For example, (S)-HPMPA and PME-5FC were nearly inactive against HIV with EC_{50} values of 77 and >100 μ M, respectively. However, their HDP- esters had EC_{50} values of 0.007 and 0.14 μ M.^{14,26} HDP-(S)-HPMPA also showed marked increases in antiviral activity versus (S)-HPMPA against vaccinia, cowpox and HCMV, in vitro.³ The hexadecyloxypropyl esters of PMEA, PMEDAP, and PME-N⁶-cyclopropyl-DAP also showed increased anti-HIV activity ranging from 5,000 to 73,000 fold versus the respective unmodified bases.²⁶ Although the HDP esters typically have greater cytotoxicity, their selectivity indexes were generally much greater due to the very large increases in antiviral activity. We conclude that esterification of acyclic nucleoside phosphonates with alkoxyalkyl or alkyl groups may be a generally useful approach for enhancing the performance of the acyclic nucleoside phosphonates.

Table 7. Antiviral activity of hexadecyloxypropyl esters of various acyclic nucleoside phosphonates against HIV-1 in vitro

Compound	Efficacy vs. HIV-1 EC ₅₀ (μM)	Fold increase	Cytotoxicity CC ₅₀ (μM)	Selectivity Index
(S)-HPMPA	77.5	—	>100	>1.29
HDP-(S)-HPMPA	0.007	11,000	1.0	143
PMEA	1.1	—	157	143
HDP-PMEA	1.5×10^{-5}	73,000	0.06	4,000
PMEDAP	0.08	—	5.3	66.3
HDP-PMEDAP	1.6×10^{-5}	5,000	0.02	1,250
PME-N ⁶ -cPr-DAP	0.07	—	4.2	60
HDP-N ⁶ -cPr-DAP	$<1 \times 10^{-5}$	>7,000	0.12	>12,000
PME-5FC	>100	—	>100	—
HDP-PME-5FC	0.14	>710	24	174

Antiviral assay: p24 reduction in MT-2 cells infected with HIV-1_{lai} as reported previously.¹²
 Adapted from Hostetler et al.¹⁴ and Valiaeva et al.²⁶

IX. CONCLUSIONS

Alkoxyalkyl esters of CDV were synthesized and evaluated in vitro in cells infected with poxviruses, herpesviruses, adenoviruses and a polyoma virus. The hexadecyloxypropyl and octadecyloxypropyl esters of CDV had >100 fold increases in antiviral activity because of increased cell uptake and conversion to the diphosphate. The intracellular half life of CDV diphosphate was 8 days. Structure activity studies showed that alkyl or alkoxyalkyl chains of 20 or 22 atoms gave optimal antiviral activity against poxviruses and herpesviruses. HDP-CDV and ODE-CDV had broad spectrum activity and were active in vitro against poxviruses, herpesviruses, adenovirus and BK virus, a polyoma virus. HDP-CDV and ODE-CDV were orally bioavailable and were active in lethal models of poxvirus and herpesvirus disease. HDP esters of (S)-HPMPA were unexpectedly active against HIV-1 and the approach appears to be generally applicable to other acyclic nucleoside phosphonates.

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CCR5 ANTAGONISTS FOR THE TREATMENT OF HIV INFECTION AND AIDS

Manos Perros

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I. INTRODUCTION

HIV replication causes AIDS, a major cause of mortality and morbidity worldwide. Since the 1980's, when the viral cause of AIDS was first discovered, academic institutions as well as pharmaceutical companies have engaged considerable resources in the discovery and development of molecules that prevent or treat the disease by blocking the replicative cycle of the virus. This resulted in an unprecedented number of effective antiviral treatments which were brought to patients within a period of 15 years from the discovery of the pathogen in the mid-80's, an enviable record of invention and a testimony to the numerous contributors.

Perhaps understandably, drug development initially targeted essential intracellular enzymes such as the polymerase, providing high confidence in the effectiveness of the blockers against the virus. At the time when the first treatments (nucleoside reverse transcriptase inhibitors, or NRTIs) became available, AIDS was an acute disease which was usually rapidly progressive and lethal. The first generation of NRTIs, requiring complex dosing schedules, was soon followed by the non-nucleoside reverse transcriptase inhibitors (NNRTIs), and in the mid-90's, the advent of the first protease inhibitors and the concept of Highly Active Antiretroviral Therapy (HAART). This brought a sea-change to the treatment of the infection, with effective combination regimens for the first time achieving impressive, sustained

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drops in viral load (VL) to levels that were undetectable even to the most sensitive technologies. Patients' immune functions were largely (albeit not completely) restored, and provided they adhered to the complex and at times unpleasant treatments, they were able to return to a near-normal day-to-day lifestyle. Pharmacological treatment had transformed AIDS from an acute, rapidly fatal condition to a chronic disease.

Despite the effectiveness of HAART regimens, and the continuous improvements made to the dosing frequency and side-effect profiles of their components,¹⁰ emergence of resistance to one or more classes of antiretrovirals remains a problem. In one large study of HIV-positive adults who received treatment yet were viraemic with >500 HIV RNA copies/mL within 3 years it was estimated that approximately 76% had resistance to one or more HIV drugs.⁷⁵ In addition, HIV resistant to one or more classes of drugs is increasingly transmitted to treatment-naïve patients, dramatically limiting their treatment options from the outset.^{52,86} This highlights the continuing unmet medical need for anti-HIV agents with novel mechanisms of action.

Until 2003, there were three classes of antiretroviral agents: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Recent discoveries elucidating the mechanism of viral entry have prepared the ground for the development of fusion inhibitors.⁷² The single currently licensed drug in this class, enfuvirtide (Fuzeon[®], Trimeris/Roche; formerly known as T-20) acts at the point of virus entry into cells by binding to the viral envelope protein gp41 and preventing a post-attachment step of the viral entry. The drug has excellent efficacy in treatment-experienced patients, but the mode of administration and cost of goods limit its use in early-stage treated or drug-naïve subjects, while resistance emerges relatively rapidly.³⁴ It has however demonstrated that HIV entry is a viable therapeutic target for future drug development, paving the way for new classes of antiretroviral agents that will benefit all patients, both treatment-naïve and treatment experienced, preserving quality of life as well as keeping subsequent therapeutic options open.

II. INHIBITION OF HIV ENTRY

The idea of blocking viral and host entry mediators to prevent initiation of a productive infection is not a new one. However for many

viruses, viral entry is mediated by host molecules that are either required in a number of physiological process in the host organism, or have a relatively low affinity and specificity raising the hurdles for the discovery of effective small molecule ligands. For instance, influenza viruses enter target cells following binding to multiple sialic acid residues that are present on either glycoproteins or glycolipids (reviewed by Skehel and Wiley⁹⁰), and are widely distributed on the cell surface, while HPV particles appear to adsorb through heparin sulfate proteoglycans present on $\alpha 6$ integrin or syndecan-1.⁸²

Luckily, HIV attaches to the cell through few specific, high-affinity molecular interactions. The process by which HIV-1 attaches to and enters host cells has been studied extensively (reviewed by Pierson et al.⁷²). The interactions between the virus and the cell surface required for entry are mediated by the viral envelope protein, Env, which consists of two surface and transmembrane subunits, termed gp120 and gp41, respectively. The gp41 protein has a glycine-rich "fusion" peptide which mediates membrane fusion, and the conformational change required for this event is the target of above-mentioned enfuvirtide. The gp120 subunit is required for specific binding to host-cell receptors. The first step in the process of HIV-1 entry is the specific binding of gp120 to CD4, the primary receptor for HIV-1, however this alone is not sufficient for HIV-1 entry.⁵⁷ The observation that the human chemokines RANTES, Mip1 α and Mip1 β are capable of inhibiting HIV-1 infection of T-lymphocytes,¹⁵ and the discovery that a human chemokine receptor (CXCR4) is an essential co-receptor for HIV-1 infection³⁰ triggered a chain reaction of discoveries: within a year a number of groups reported the discovery of a second HIV coreceptor, CCR5.^{2,13,19,21,25}

While conceptually all molecules required for effective viral entry constitute targets for the development of new antiretrovirals, many of them present significant challenges. Firstly, Env is the most sequence-variable of the HIV-1 proteins. Hence, differences in drug sensitivity between HIV-1 strains are possible, as has been described for the gp120 inhibitor, BMS-378806, which has a wide range of activities against panels of B-clade and non-B clade viruses.⁴⁹ Furthermore, targeting host receptors may inhibit their natural function. If this interrupts essential host processes then compounds may have unwanted secondary effects, as has been shown for the CXCR4 antagonist, AMD3100.³⁷

The inhibition of binding of gp120 to CD4 has been explored with mixed results. PRO-542 (Progenics Pharmaceuticals, USA), a tetravalent CD4-IgG2 fusion protein⁴¹ is now on hold in Phase II clinical development while TNX-355 (Tanox, USA), a humanized anti-CD4 antibody⁴⁶ must be injected every two weeks and is only investigated

in treatment-experienced patients where it shows a modest (0.7–0.9 log₁₀) drop in VL at 48 weeks (Tanox Press Release, May 2006). Two small molecule inhibitors, BMS-378806 and BMS-488043 (Bristol-Myers Squibb, USA), target gp120 rather than CD4.⁵⁶ Both compounds demonstrate variable activity when tested *in vitro* against panels of virus isolates,^{49,50} although BMS-488043 has shown efficacy in short-term monotherapy in HIV-1 infected individuals despite the absence of consistency amongst objects.³⁵ An analogue of BMS-378806 is being developed as an intravaginal gel to protect women from HIV infection (International Partnership for Microbiocides, company communication) following reports of efficacy in a preclinical primate model.¹⁰⁴

III. CCR5 AS A DRUG TARGET

To meet the exacting requirements of convenience and tolerability that will ensure high quality of life for HIV patients on life-long treatment, most pharmaceutical companies are now aiming for once- or twice-daily orally bioavailable molecules. G-protein coupled receptors (GPCRs) have historically been, and continue to be, attractive drug targets for several reasons. First, their location on the cell surface makes them readily accessible to drugs. They are also ubiquitous, being involved in regulation of every major mammalian physiological system. Consequently, more than 30% of about 500 commercially available drugs work by selective modulation of a GPCR¹¹¹ and new agents are constantly emerging to target GPCRs involved in a variety of disease processes.

Chemokine receptors are a large family of GPCRs that regulate leukocyte activation and migration to sites of inflammation via interaction with a family of secreted chemo-attractant cytokines or “chemokines”.¹² Perhaps unsurprisingly, the chemokine system is a prime target for microbial manipulation, and HIV is not the only virus subverting its elements to accomplish its function (reviewed by Lusso⁵⁵). The chemokine receptor network is highly redundant (>45 chemokines promiscuously recognise ~20 receptors), with most ligands able to recognise a number of alternative receptors, and each receptor having more than a single ligand.

The chemokine receptors most commonly utilised by HIV-1 *in vivo* are CCR5 and/or CXCR4.^{13,19,25,30} The preference of a given viral strain for one or the other of the two receptors is referred to as “tropism”, and the strains classified as CCR5-tropic (R5), CXCR4-tropic (X4) or dual-tropic (R5X4) (now thought to be more likely due to

circulating mixed population of R5 and X4 viruses) on the basis of their ability to enter target cells through the corresponding receptor(s). Of the two HIV co-receptors, CCR5 appears the most suitable candidate target for novel therapeutics.

Much of the early rationale behind the development of CCR5 inhibitors was provided by the discovery of a naturally-occurring 32-bp deletion (CCR5- Δ 32) within the coding sequence of the gene which results in translational frame-shift, resulting in an aberrant, truncated protein. The product of the defective allele is incorrectly folded and is not transported to the cell surface. Subjects (approximately 1% of the Caucasians) who are homozygotes for this deletion are genetically protected from HIV infection^{17,39,54,78} while heterozygotes have delayed disease progression,^{17,39} which does not appear to be consistently associated with decreased viral load, and raises the question of a role for the receptor in viral pathogenesis post-entry.^{79,95} The absence of an overt phenotype associated with CCR5- Δ 32 homozygosity provided considerable support to the confidence in safety of a CCR5 antagonist. Finally, CCR5-tropic viral strains are the most commonly transmitted,^{80,83,114} they usually predominate throughout asymptomatic infection and approximately 50% of patients who develop AIDS carry only R5 strains.^{44,45}

More recently, several CCR5 antagonists have been shown to be effective inhibitors of HIV-1 infection in humans.^{29,47}

CCR5 is a 352-amino-acid protein with a basic structure that is typical of a seven-transmembrane G protein-coupled receptor (GPCR). It consists of three extracellular loops (ECL1, ECL2 and ECL3) and the N terminus, which are involved in chemokine binding and interactions with the viral protein gp120, and three intracellular loops and the C terminus, which participate in G protein-mediated signal transduction. The activation of CCR5 by its endogenous ligands activates cellular responses including inhibition of cAMP production, stimulation of Ca^{2+} ion release and activation of MAP kinase and Jun-N-terminal kinase.⁹⁴ The endogenous ligands of CCR5 include the chemokines known as macrophage inflammatory protein (MIP)-1 α , MIP-1 β and "regulated upon activation, normal T-cell expressed and secreted" (RANTES) (reviewed by Mueller and Strange⁶⁶). It is worth noting that RANTES and (MIP)-1 α can recognise multiple chemokine receptors in addition to CCR5, in contrast to CXCR4 that has an exclusive 1–1 association with SDF1- α . The observation that CCR5 ligands can inhibit HIV-1 entry¹⁵ led to the identification of CCR5 as a co-receptor for HIV-1.

The physiological function of CCR5 in a fully developed organism remains to be elucidated. While it is reassuring that Δ -32 homozygotes have no major apparent deficiencies, there remains a possibility that inhibition of CCR5 in a fully-matured immune system will have a different effect to the constitutive knock-out that allows the organism to utilise redundancies in order to compensate for the loss of the receptor during pregnancy and early childhood. A better understanding of the physiological role of CCR5 may also provide alternative development indications for CCR5 inhibitors, possibly against one or more of the pathologies that appear to be negatively associated with CCR5 knock-out or inhibition, including primary sclerosing cholangitis^{27,36} rheumatoid arthritis,^{73,74} atherosclerosis,^{9,103} hepatitis C-associated lesions^{32,105} and cardiac transplant rejection.^{18,31}

IV. THE DISCOVERY OF NOVEL CCR5 ANTAGONISTS

Aminergic GPCRs are tractable, and their ligand-binding domain is configured to recognise small basic drug-like molecules. By contrast, CCR5 and CXCR4 recognise short polypeptides, which bind the receptor through a complex, multi-stage process, involving both extracellular and transmembrane domains.⁶⁷ Additionally, low molecular weight inhibitors cannot readily be designed by analogy to the natural receptor ligands, and discovery of lead matter has to rely on random high-throughput screening (HTS). Under the circumstances, biologicals (such as antibodies) are an appealing alternative, and an early drug candidate, PRO-140 (Progenics Pharmaceuticals, USA), a murine monoclonal antibody spanning multiple extracellular domains of CCR5^{69,100} is currently in clinical development in HIV patients. Despite the promising pharmacokinetics indicating potentially once-monthly administration, the molecule has to be injected intravenously while long-term safety and efficacy remain to be established.⁶⁸

Despite the challenges and risks outlined above, a number of companies launched small-molecule discovery programmes. Given the limited throughput of the majority of antiviral assays available in the mid-90s, developing a high-throughput screening technology capable of rapidly evaluating thousands of compounds and providing short turnaround times for SAR (structure–activity relationship) generation would prove a valuable advantage. Radioligand-binding assays, using iodinated chemokines¹⁶ were used successfully by Takeda, Merck, Schering, ONO and Pfizer to identify initial chemical leads.^{4,11,23,59,87,92} To study the interaction of the viral envelope

protein with CCR5, a number of investigators described binding assays between purified iodinated^{99,112} or biotinylated⁶⁹ gp120 protein and recombinantly expressed receptor on either intact cells or membrane extracts. While those assays provided essential insights into the mechanisms of viral attachment, expression and purification of fully-glycosylated functional gp120 were proving particularly challenging and costly to scale-up. To circumvent this obstacle, Dobbs and collaborators^{20,76} measured binding of gp120:CD4 complexes to CCR5 using a Europium-labelled anti-gp120 monoclonal antibody, followed by time resolved fluorescence (TRF). The ability of cyanovirin-N (CV-N) to interact with the oligosaccharide groups on recombinant soluble gp120 was similarly used to develop a TRF assay.⁶² These assays were run in a 96-well format, making them suitable for targeted screening of small chemical libraries. Technologies with still higher throughput potential were developed more recently using the ability of recombinant Env to induce membrane fusion when expressed on the cell surface. A key hurdle in developing fusion assays as screens, is obtaining cell surface expression of Env. A number of groups have developed assays where the protein is transiently expressed. One of the most elegant solutions utilises beta-galactosidase alpha-complementation activity to detect fusion events occurring between transiently-transfected cell lines.³⁸ BacMam baculovirus was also used to optimise transient transduction of HEK293 cells with Env, Tat and Rev, resulting in a high-throughput 96-well assay.⁴² However transient transfection systems are not ideal for large screening campaigns both in terms of cost as well as reproducibility. Litwin et al.⁵³ used fluorescence resonance energy transfer (FRET) measurements to quantify fusion events between Env-expressing HeLa cells and CCR5-expressing PM-1 which had previously been loaded with fluorescent dyes F18 and R-18 respectively. More recently, a 384-well assay was developed using Chinese Hamster Ovary (CHO)-Tat10 and HeLaP4 cell lines. The CHO-Tat10 cell line was engineered to constitutively express Env and the Tat transactivator protein while HeLaP4 co-express CD4 and CCR5, as well as the beta-galactosidase reporter under the control of the HIV-1 long terminal repeat (LTR). Fusion between the two cell types resulted in transactivation by Tat of the HIV-1 LTR and expression of the reporter protein. This technology has the added advantage of detecting not only inhibitors of HIV entry, but also compounds that block Tat-induced TAR transactivation.⁷ Finally, earlier this year was reported an assay able to detect both CCR5- and CXCR4-dependent fusion utilising inducible Env constructs.⁴³ The reported correlation between activity in the above fusion assays and *in vitro* antiviral activity is excel-

lent ($r^2 > 0.8$)⁷⁰ which highlights a key advantage for this approach over ligand-binding screens for identifying lead co-receptor antagonist molecules with antiviral activity.

While the above assays can be excellent first-line screens, they cannot easily be modified to determine the activity of the investigational blockers against a range of viral envelope sequences from various clades, which given the inherent variability of Env is a requirement. In the last few years the armamentarium of the above surrogate assays was complemented by efficient single-cycle envelope-recombinant infectious assays (reviewed in Coakley et al.¹⁴), and more recently a fully-replicative high-throughput virus replication assay.⁶ Today we are certainly a far cry from 24-well antiviral assay technology that was widely used when most discovery programmes were initiated in the mid-'90s.

Despite the technological challenges at the time, less than three years passed between the initial description of the role of CCR5 in HIV replication and the first small-molecule CCR5 antagonists reported by Takeda Chemical Industries (Japan).⁴ Although active against R5 HIV-1 *in vitro* ($EC_{50} < 4$ nM), TAK-779 (Figure 1) development was discontinued most probably due to formulation problems and lack of oral bioavailability. Takeda then began development of TAK-220 (Figure 1), which inhibits binding of MIP-1 α to CCR5, but not MIP-1 β , and is orally bioavailable in rats and monkeys.^{40,93} TAK-220 inhibited the *in vitro* replication of R5 HIV-1 clinical isolates, with a mean IC_{90} of 13 nM, and was reported to have positive antiviral interactions with a range of antiretroviral drugs *in vitro*.⁹⁷ The most recent developments at Takeda involve another candidate CCR5 antagonist, TAK-652 (Figure 1). The compound inhibited the binding of RANTES, MIP-1 α , and MIP-1 β to CCR5-expressing cells at nanomolar concentrations. TAK-652 also suppressed the binding of MCP-1 to CCR2b-expressing cells with an IC_{50} value of 5.9 nM. The compound slightly inhibited the ligand-binding to CCR3- and CCR4-expressing cells but did not affect the ligand-binding to CCR1- and CCR7-expressing cells even at micromolar concentrations. Consistently with its mode of action, TAK-652 selectively inhibited R5 HIV-1 but not X4 HIV-1 replication. Furthermore, TAK-652 inhibited the replication of 6 R5 HIV-1 clinical isolates, including reverse transcriptase- and protease inhibitor-resistant mutants, in PBMC with a mean EC_{90} value of 0.25 nM. In addition, all recombinant HIV-1 strains with seven different subtype (A to G) envelope proteins were equally susceptible to TAK-652 with a mean IC_{50} value of 1.0 nM. The anti HIV-1 activity of TAK-652 decreased five-fold with high concentrations of human serum. Single oral administration

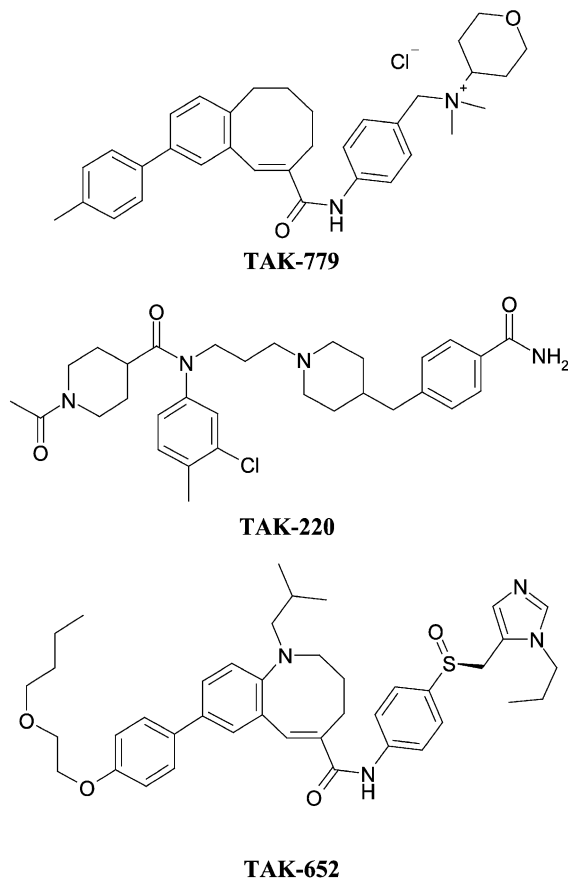


Figure 1. Chemical structures of the Takeda CCR5 antagonists (Takeda Chemical Industries, Japan).

(25 to 100 mg) of TAK-652 solution was safe and well tolerated. TAK-652 showed good oral absorption, and its plasma concentration at 24 h after administration (25 mg) was 7 ng/mL (9.1 nM).⁵ TAK-652 has also demonstrated favourable *in vitro* antiviral interactions with a range of marketed antiretroviral drugs.⁹⁶

Merck have provided detailed structure activity relationships of a series of agents with CCR5 antagonist activity.^{22,84,85,88} Replacement of an earlier pyrrolidine group (1, Figure 2) by tri-substituted cyclopentanes has resulted in the compound known as CMPD-167 (Figure 2). This was shown to protect macaques from infection by an R5 strain

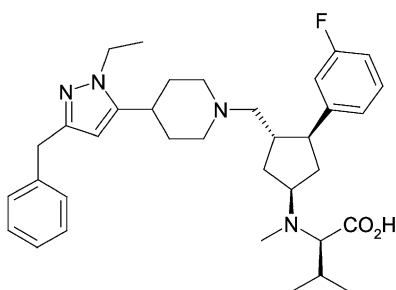
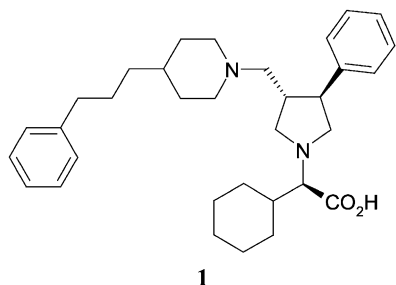


Figure 2. Chemical structures of the Merck CCR5 antagonists (Merck, USA).

of SHIV,¹⁰⁴ and was subsequently licensed to International Partnership for Microbiocides where it is being developed as an intravaginal gel to protect women from HIV infection. In a recent communication, what appears to be the same molecule renamed MRK-167 was administered (5 and 10 mg/kg) to monkeys undergoing transplantation, where it appeared to block post-surgical hyperthermia with the lower dose affecting inhibition of CCR5/CD68+ cell migration to the allograft. Modest improvements in survival time were also observed. A synergistic effect was seen on administration of MRK-167 with cyclosporin A.¹⁸

Schering-Plough has also been developing CCR5 antagonists for a number of years. Its first major candidate, SCH-C (SCH-351125; [Figure 3](#)), was the first CCR5 antagonist to be studied for clinical efficacy. SCH-C is a small oxime-piperidine derived from a compound identified by high throughput screening of inhibitors of RANTES-CCR5 binding. *In vitro* infectivity assays using SCH-C and primary R5 HIV-1 isolates demonstrated a mean IC_{50} of less than 9 nM, while potent *in vivo* activity was demonstrated in SCID-hu Thy/Liv mice.⁹² Although a study in 12 HIV-infected patients demonstrated good oral bioavailability and

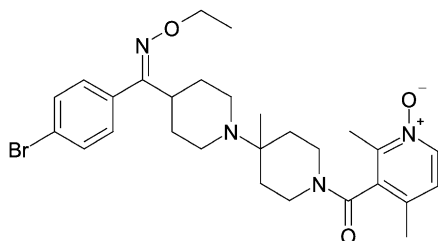
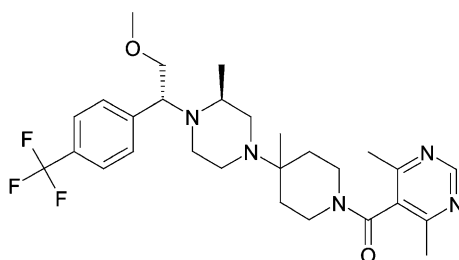
**SCH-C/SCH-351125****Vicriviroc (SCH-D/SCH-417690)**

Figure 3. Chemical structures of the Schering CCR5 antagonists (Schering-Plough Corporation, USA).

a 3-fold or greater reduction in viral load at 25 mg BID, observations of QTc prolongation in healthy volunteers receiving 600 mg QD led to the termination of further studies of SCH-C.²⁸ Schering-Plough has continued to develop agents related to SCH-C, most notably vicriviroc (SCH-D, SCH-417690; [Figure 3](#)), which is reported to be more potent than SCH-C and has a more favourable safety profile with respect to QTc prolongation.⁹¹ The compound was originally studied in both treatment-naïve and treatment-experienced patients in combination with combivir (zidovudine 300 mg + lamivudine 150 mg once daily, GlaxoSmithKline). However the treatment-naïve study was discontinued upon the recommendation of an independent data safety monitoring board (DSMB) due to virological breakthrough. The data from the study was disclosed recently.³³ Primary analysis at 2 weeks showed a mean decrease in HIV RNA of up to 1.34 log 10 in the 75 mg arm. Despite good tolerability and a clear response to treatment at two weeks, vicriviroc in combination with combivir failed to sustainably suppress viral load, as evidenced by significantly higher viral breakthrough (RNA \geq 50 copies/mL) at all doses (22% in the 75 mg

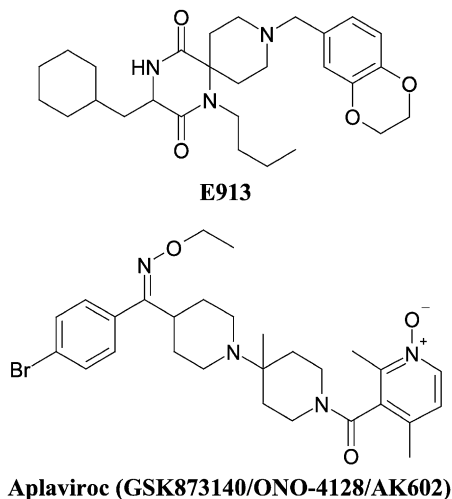


Figure 4. Chemical structures of the Ono/GSK CCR5 antagonists (GlaxoSmithKline, UK and Ono Pharmaceuticals, Japan).

arm) relative to efavirenz (8%), most likely accounting for the original DSMB recommendation. In a more recent review of the ongoing trial in treatment-experienced subjects, a study-monitoring committee identified four cases of lymphoma and one of gastric adenocarcinoma, however the trial is continuing because the drug has shown antiviral activity and the AIDS Clinical Trials Group (ACTG), which is conducting the study, has been unable to find a causal link between the drug and the malignancies.

Until October 2005, GlaxoSmithKline (GSK) was also developing a small-molecule CCR5 antagonists based on spirodiketopiperazine (SDP) derivatives previously reported by Ono Pharmaceuticals (Japan) (E913, [Figure 4](#)).⁶⁰ This class of compounds originated from a generic template based on 1,4,4-trisubstituted-piperazines, which have activity against a range of GPCRs, and were subsequently optimised through parallel chemistry. Aplaviroc (GSK873140, ONO-4128, AK-602) (licensed from Ono Pharmaceuticals in January 2003; [Figure 4](#)) is effective at inhibiting primary HIV-1 isolates *in vitro* with an IC_{50} as low as 0.2 nM without blocking RANTES or MIP-1 β binding, yet it does inhibit RANTES-mediated signalling and chemotaxis.^{59,107} In a Phase IIa clinical study, patients receiving 600 mg of aplaviroc BID for 10 days experienced a mean viral load reduction from baseline of 1.66 log₁₀ copies/mL.⁴⁷ While upon short-term administration the drug ap-

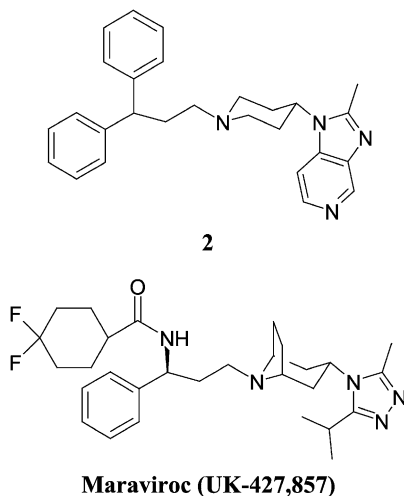


Figure 5. Chemical structures of the Pfizer CCR5 antagonists (Pfizer Global R&D, UK).

peared to be well tolerated, apart from mild/moderate gastrointestinal side-effects including nausea, vomiting and abdominal pain, chronic administration produced cases of severe liver toxicity, leading to the early termination of the Phase IIb studies in treatment-naïve patients in September 2005. A further case of elevated levels of liver enzymes and bilirubin in a treatment experienced patient from the Phase III trial put an end to all development activities on aplaviroc. Hepatotoxicity is the most frequent cause of compound attrition in development (in preclinical toxicology in particular), and there are no physiological indications that CCR5 could be involved in liver function. The absence of drug-related reported hepatotoxicity either in pharmacologically valid preclinical models or in man with either of the other advanced CCR5 antagonists comforts the hypothesis that the above findings were compound specific rather than due to CCR5 blockade.

Pfizer's CCR5 antagonist maraviroc (UK-427,857; [Figure 5](#)) appears to be the only remaining small molecule inhibitor in development for both treatment-naïve and treatment-experienced patients. The compound evolved from a series of substituted piperidines (**2**, [Figure 5](#)) which were discovered by high-throughput screening. The medicinal chemistry programme which led from the original hit to maraviroc resulted in the conformational restriction of the piperidine into a tropane, and the introduction of a phenylacetamide group which appears to be essential for antiviral activity.³ Maraviroc has

nanomolar potency against chemokine binding and signalling (measured by Ca^{++} -flux as well as GTP- γ S) and is exquisitely selective for CCR5, with no activity at micromolar concentrations against any other chemokine receptor, as well as a range of relevant enzymes, receptors and ion channels, including the human Ether-a-Gogo related gene (hERG) which is thought to be involved in QTc prolongation. It has broad-spectrum antiviral activity *in vitro* against primary strains from all clades and from various geographic locations, with a mean EC_{90} of 2 nM. Maraviroc was also tested against 200 clinically derived HIV-1 envelope-recombinant pseudotyped viruses, 100 of which were derived from viruses resistant to existing drug classes.²³ When tested in combination with a number of licensed or investigational drugs from all classes (including fusion inhibitors) maraviroc was found to be additive to slightly synergistic.⁶³ Pharmacokinetic studies have demonstrated good oral bioavailability and a terminal half-life of 16–23 hours following multiple dosing, indicating that the compound has potential for once-daily dosing.^{1,106} Single doses of up to 900 mg and multiple doses of up to 300 mg BID for 28 days were well tolerated.^{1,77} In Phase IIa studies, treatment-naïve HIV patients who received maraviroc monotherapy at doses ranging from 25 mg QD to 300 mg BID for 10 days experienced a viral load reduction of 1.64 \log_{10} copies/mL at day 10 and a maximum VL reduction of 1.84 \log_{10} copies/mL at the top dose. The drug appears to be well tolerated with headache, nausea and flatulence being the only adverse events occurring at a rate higher than placebo at doses up to and including 300 mg BID. Pfizer is currently pursuing Phase IIb/III trials with maraviroc, three studies in treatment-experienced and one in treatment-naïve patients with evaluation of viral tropism¹⁴ at pre-screening and during follow up. The trials were designed to compare the efficacy of once-daily or twice-daily maraviroc *vs* efavirenz in a combivir background in naïve patients, or maraviroc plus optimised background therapy (OBT) *vs* placebo plus OBT in treatment-experienced patients. All studies are still ongoing and blinded, although an independent DSMB review resulted in early termination of a 300 mg QD arm in the treatment-naïve study as it failed to meet predefined non-inferiority criteria against the standard-of-care efavirenz. Isolated cases of liver dysfunction have been reported on maraviroc in the presence of other known hepatotoxic drugs or injections, and a causal relationship has been deemed unlikely. The compound has received fast track designation by the FDA.

V. MOLECULAR MECHANISMS

A converging stream of evidence indicates that in order to exert antiviral activity, CCR5 antagonists bind at an allosteric site. Indeed, a number of site-directed mutagenesis studies^{11,24,26,58,81} have positioned the binding site for the small-molecule antagonists in a lipophilic pocket modelled on the transmembrane domain of rhodopsin, while interaction of gp120 with the receptor occurs on the surface (N-terminus and ECL2, [Figure 6](#)).^{48,67,89,113} The allosteric mode of inhibition is also consistent with the findings from combinations studies of aplaviroc with SCH-C, vicriviroc, TAK-779,^{102,107} and with the apparent “non-competitiveness” and slow-offset of those molecules from CCR5.^{23,107} While a single residue (E283) in the transmembrane region of CCR5 appears to be pivotal for all CCR5 inhibitors tested (probably serving as an “anchor” for the basic centre common to all the molecules disclosed to date), mutagenesis at other sites surrounding the putative binding pocket results has different effects.^{24,81} This suggests that the antagonists interact differentially with the transmembrane helices and ECLs, producing distinct conformations of the receptor.⁵⁸ With hindsight, this is probably the most likely way in which a low molecular weight antagonist can effectively block a protein–protein interaction occurring over larger surface areas and involving a number of high-affinity interactions. In addition to the biochemical rationale, there is also a biological argument in favour of a slowly-reversible or irreversible allosteric antagonist, as the conformational changes that follow gp120 binding to CCR5 lead to membrane fusion and are therefore practically irreversible; a rapidly reversible competitive inhibitor would be at a kinetic disadvantage to the virus and antiviral activity would have been unlikely.

One of the most interesting consequences of the allosteric binding mode is the potential for favourable pharmacodynamics. In monotherapy trials of CCR5 antagonists the rebound in viral load after treatment discontinuation does not appear to be immediate, the delaying varying from 1 to 2 days for vicriviroc³³ and aplaviroc⁴⁷ to up to 5 days for maraviroc.²⁹ Pharmacokinetic persistence may account for the 1–2 day delay seen with vicriviroc, which has a plasma half-life of approximately 24 hours, however for maraviroc, a terminal plasma half-life of 16–23 hours is not sufficient to account for a 5 day prolongation of virologic response. Receptor occupancy remained high (>60%) 5 days after maraviroc discontinuation at all effective doses,²⁹ consistently with the data generated *in vitro*.²³ Surprisingly therefore, and despite high CCR5 occupancy (95%) by aplaviroc, with an estimated half-life of be-

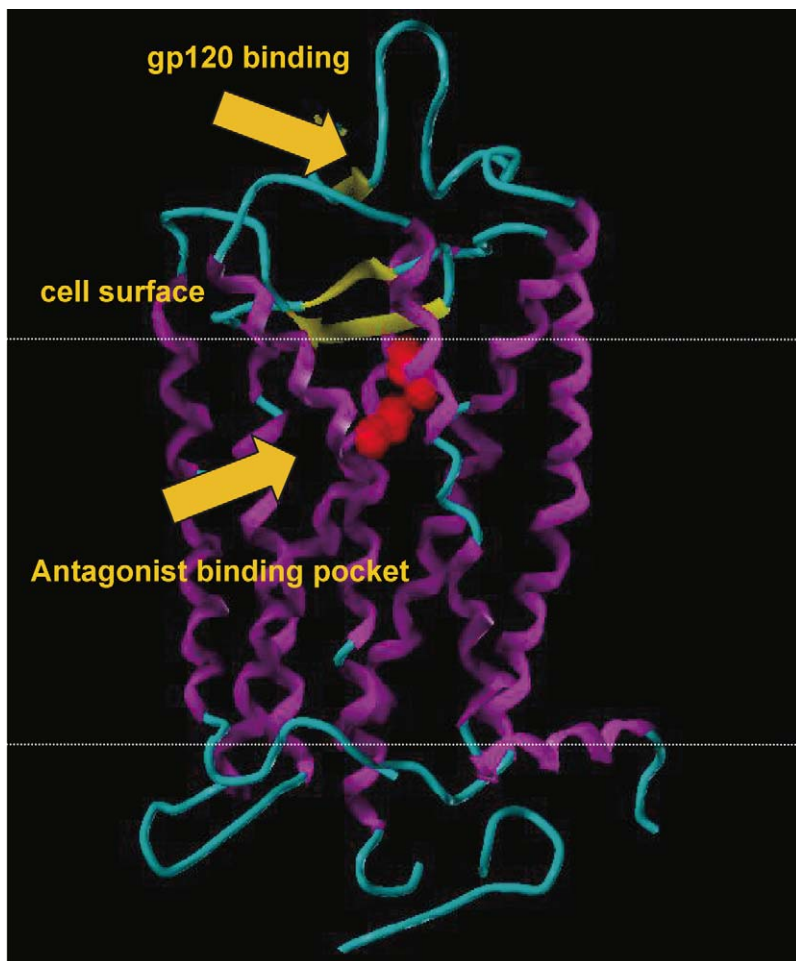


Figure 6. Proposed binding site for allosteric antagonists on CCR5. The model is based on site-directed mutagenesis data and modelling from the 3D structure of the bovine rhodopsin transmembrane region. A basic small-molecule ligand is pictured in the proposed binding site.

tween 69 and 152 hours, the relative efficacy of once-daily versus twice-daily monotherapy was more in-line with its short pharmacokinetic half-life.⁴⁷ The complexity and the turnover of both the immune target cells and the receptor available on their surface is probably responsible for this apparent discrepancy. A combination of long plasma half-life and slow receptor off-rate is likely to be required for optimal efficacy.

VI. RESISTANCE TO CCR5 ANTAGONISTS IN VITRO AND IN THE CLINIC

Another interesting consequence of the allosteric binding mode of CCR5 antagonists is the way in which virological resistance occurs. Emergence of resistance is the greatest challenge for all antiretroviral classes of drugs. The problem is usually addressed by achieving high multiples of efficacious concentrations, however CCR5 antagonists face a particular risk. As CCR5 is a host target, and therefore does not mutate, the virus needs to develop the ability to replicate in the presence of receptor-inhibitor complex, as opposed to developing more typical antiretroviral resistance which occurs by generation of viral mutants that can exclude the inhibitor from its target site. There are theoretically two distinct ways in which the virus could develop resistance to a CCR5 antagonist: one consists of developing the ability to utilise antagonist-bound receptor, the second is to utilise CXCR4 as a coreceptor. One of the earliest studies of the development of resistance to CCR5 inhibitors reported R5 virus switching tropism.⁶⁴ The findings of that study may not be relevant to treatment with small-molecule CCR5 antagonists, since the agents used by Mosier and colleagues were analogues of the agonist, RANTES that are known to induce receptor internalisation, making receptor tropism shift the only possible route to resistance. In contrast, there have been several independent observations *in vitro* of the selection of virus that is resistant to a CCR5 antagonist but has retained CCR5 tropism.^{59,61,101,110} In all cases, resistance appears to emerge slowly, and require multiple mutations in the variable regions of the viral envelope proteins. Encouragingly, maraviroc-resistant strains retain sensitivity to other CCR5 antagonists¹⁰⁹ indicating that not only do they still rely on CCR5 for entry, but their ability to utilise antagonist-associated receptor is limited to a single compound, maraviroc. Incidentally, should this *in vitro* observation translate to the clinic, patients failing maraviroc may still have further clinical options within the same class. It appears therefore that by allowing the virus to recognise an alternative configuration of CCR5 on the cell surface, allosteric inhibitors prevent generation of CXCR4-tropic virus *in vitro* and reduce the likelihood of rapidly emerging class resistance.

Emergence of X4 variants during CCR5 antagonist treatment *in vivo* may be therefore be more likely from pre-existing reservoirs than via mutation of circulating R5 virus.

The emergence of X4 virus during treatment with CCR5 antagonists has been described for a minority of treated patients undergoing

short-term monotherapy. In the Phase IIa trials of maraviroc CXCR4-tropic virus was detected in 2 of 63 patients with CCR5-tropic virus at baseline following 10 days of monotherapy. Phylogenetic analysis indicated that the CXCR4-using variants probably emerged from a pre-existing CXCR4-using reservoir, rather than via co-receptor switch of a CCR5-tropic clone under selection pressure from maraviroc.¹⁰⁸ Similar findings have been reported for vicriviroc and aplaviroc. The clinical relevance of these findings in the context of an optimized multi-drug regimen is not clear. In some individuals, CXCR4-tropic virus emerges over time, and this has been associated with rapid CD4⁺ T-lymphocyte decline and accelerated disease progression. Although increasing prevalence of X4 virus and decreasing prevalence of R5 virus have been associated with increasing viral load and decreasing CD4 cell counts,^{8,65} a switch to CXCR4-tropism is not exclusively required for the development of AIDS. Throughout infection, the detection of R5 virus only is most common, although dual/mixed-tropic virus is more likely to be detected in advanced patients. The detection of exclusively X4 virus is rare.⁶⁵ Whether emergence of X4 strains is a marker for disease progression rather than the cause may only be answered following carefully controlled multi-centre clinical trials of CCR5 antagonists, such as those discussed above.

VII. CONCLUSIONS

The discovery of the HIV coreceptors CCR5 and CXCR4 provided scientists with exciting new targets for the development of new antiretrovirals. Epidemiological data from the naturally occurring Δ -32 mutation provided early confidence in rationale and safety for CCR5 antagonists, attracting the attention and contributions of a number of academic institutions and pharmaceutical companies. But the path leading to potential new therapies has been unexpectedly treacherous. From a number of molecules that once held promise as future therapeutics only a handful remain in development today. Early chemical lead matter has been relatively easy to find (most chemical files being rich in GPCR pharmacophores) but development of orally bioavailable, selective clinical candidates has proved more challenging. To gain affinity and develop potency, most of the current molecules are pushing the boundaries of the “rule of 5” both in terms of potency and potential hydrogen bond-forming groups, thus restricting oral bioavailability.⁵¹ Their basic lipophilic core appears to be an ideal pharmacophore for the hERG channel.⁷¹ Blockade of hERG function is the most common cause

of long QT syndrome, a disorder of cardiac repolarisation that predisposes patients to potentially fatal arrhythmias. This syndrome was the demise of the first CCR5 antagonist ever to be tested in the clinic, SCH-C, and in the author's own experience, gaining selectivity against this channel while retaining all other desirable properties has been one of the most challenging medicinal chemistry problems. Ever developing screening technologies enabled large-scale parallel chemistry to develop SAR, and the optimisation of single-cycle viral replication assays provided researchers with a readily tool to evaluate antiviral potency, and clinicians with a rapid assay to assess viral tropism. Despite the numerous challenges, only 10 years have passed since the initial discovery of the HIV coreceptors, and with two molecules in advanced clinical development, there is hope that a new treatment may become available within the next few years.

Perhaps in this future new class more than in others, there is scope for more new drugs. The allosteric mode of action of those inhibitors suggests that it should be possible to create a wide variety of CCR5 conformations by altering the way in which the compounds interact within their binding cavity, and resistant virus should only recognise one of many possible antagonist-bound configurations. While it will most likely be impossible to ever combine two CCR5 antagonists in a single regimen with substantial benefit to the patient, we have established that *in vitro* generated maraviroc-resistant viruses are sensitive to other CCR5 antagonists. It would be of great interest to see if this type of resistance also develops in the clinic. Should this be the case, it is a strong possibility that sequencing therapies within the class will not lead to an ever-decreasing efficacy against the viral population that gradually develops class resistance. Given the rapid variability of the viral envelope and the findings described from *in vitro* systems, it may even be possible to re-use earlier treatments without significant loss of efficacy. A further avenue worth exploring is that of combining CCR5 antagonists with fusion inhibitors that act on the other steps of the viral entry,^{63,96–98} turning the highly synergistic nature of the process to the physician's advantage. And of course, while the compounds currently in clinical development may be a quantum leap, further improvements in toleration and compliance can be achieved by further reducing synthetic complexity (an important consideration for expanding access globally), reducing dose size and dosing frequency, and ensuring that there are no P450 interactions to interfere with combinations to existing agents. While a synthetically tractable, sub-100 mg, well-tolerated once-daily antiretroviral may not be an easy

objective to achieve, CCR5 antagonist chemotypes certainly offer a more amenable substrate than most traditional classes.

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THE MEDICINAL CHEMISTRY OF THE DATA AND DAPY SERIES OF HIV-1 NON-NUCLEOSIDE REVERSE TRANSCRIPTASE INHIBITORS (NNRTIS)

Jan Heeres and Paul J. Lewi

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ABSTRACT

This contribution presents an historical account of the medicinal chemistry of the DATA (diaryltriazine) and DAPY (diarylpyrimidine) series of inhibitors of HIV-1 reverse transcriptase (NNRTIs). The history extends over a period of more than 18 years, starting from 1987, and is still ongoing. The authors attempt to describe the often tortuous and unpredictable route that led from the virological screening of compounds, over serendipitous chemistry, X-ray crystallography, molecular modelling, animal pharmacokinetics, biochemistry and physical chemistry to the design and further development of highly potent anti-HIV compounds, three of which are now in advanced stages of clinical studies. The emphasis in this historical account is on the medicinal chemistry that led to the DATA and DAPY series of NNRTIs.

*The authors dedicate this contribution to the memory of
Dr. Paul Janssen (1926–2003), founder of Janssen Pharmaceutica NV
and inventor of medicines*

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I. INTRODUCTION

Many people have contributed in important ways and over many years to the design and development of the DATA and DAPY series of HIV-NNRTIs. This would not have been possible, however, without the leadership, tenacity, intuition, and knowledge of medicinal chemistry of the late Dr. Paul Janssen. The authors had the privilege to work with “Dr. Paul” on the medicinal chemistry of the DATA and DAPY compounds during the last eight years of his life at the Center for Molecular Design in Vosselaar (Belgium). This research was carried out in collaboration with scientists from Janssen Pharmaceutica, Tibotec and with external collaborators from many parts of the world. It is the result of accumulated experience over almost two decades of medicinal research in the field of HIV.

A strategic collaboration between Janssen Pharmaceutica (Beerse) and the Rega Institute for Medical Research (Leuven), which started in 1987, gave rise to the evaluation of part of the Janssen compound collection against HIV, the causative agent of AIDS. Very soon hit compounds with specific HIV-1 activity were identified. Lead optimisation finally resulted in the discovery of the TIBO series^{22,15,16} and the α -APA series,^{23,6} represented by compounds **1** and **2**, respectively (Figure 1).

Both series of compounds appeared to inhibit HIV-1 reverse transcriptase (RT), an essential enzyme for the replication of the virus. A few years later it became known from X-ray crystal structure studies of the ligand–enzyme complex that these classes of non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind in a flexible allosteric binding site of reverse transcriptase at a distance of approximately 10 Å from the polymerizing processing site.^{14,6,7} Subsequent research in this domain resulted in the synthesis of the ITU-series, of which the best compounds were active in the single digit nanomolar range (Figure 2).¹⁹

Initially, the virological panel included the wild type HIV-1 LAI virus (wt, also referred to as IIIB), four recombinant single mutants (L100I, K103N, Y181C and Y188L) and two recombinant double mutants (L100I + K103N and K103N + Y181C). From 1994 on, the virological screening of Janssen compounds was continued at Tibotec (first in Antwerp, then in Mechelen). The virological screening panel at Tibotec was extended considerably in the course of time. (For reasons of consistency, however, we limit our discussion of the virological activity spectrum of NNRTIs against mutant viruses to the initial screening panel.)

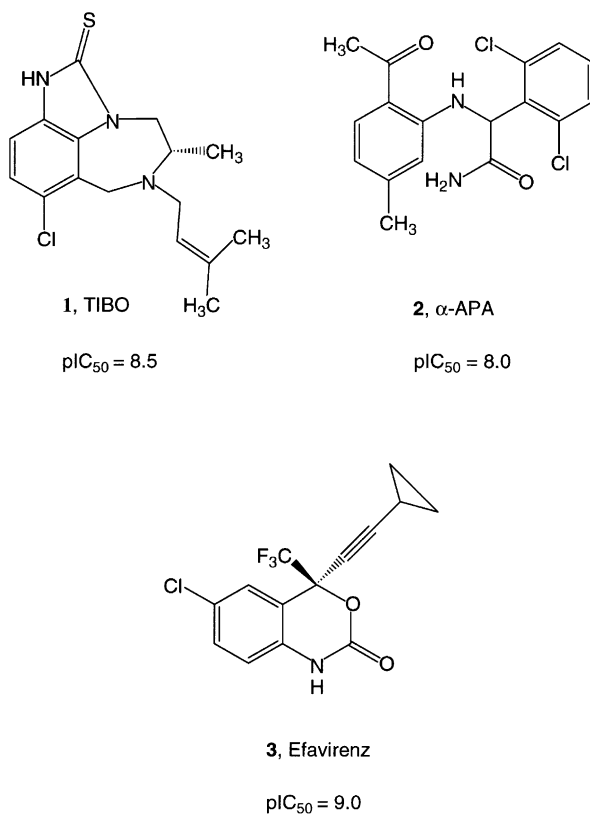


Figure 1. Structural formulas of the prototype TIBO, α -APA and the reference compound efavirenz, together with their respective pIC_{50} values for inhibition of wild type HIV-1 replication.

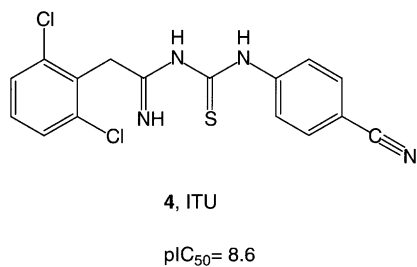


Figure 2. Prototype ITU compound and pIC_{50} for inhibition of wild-type HIV-1 replication.

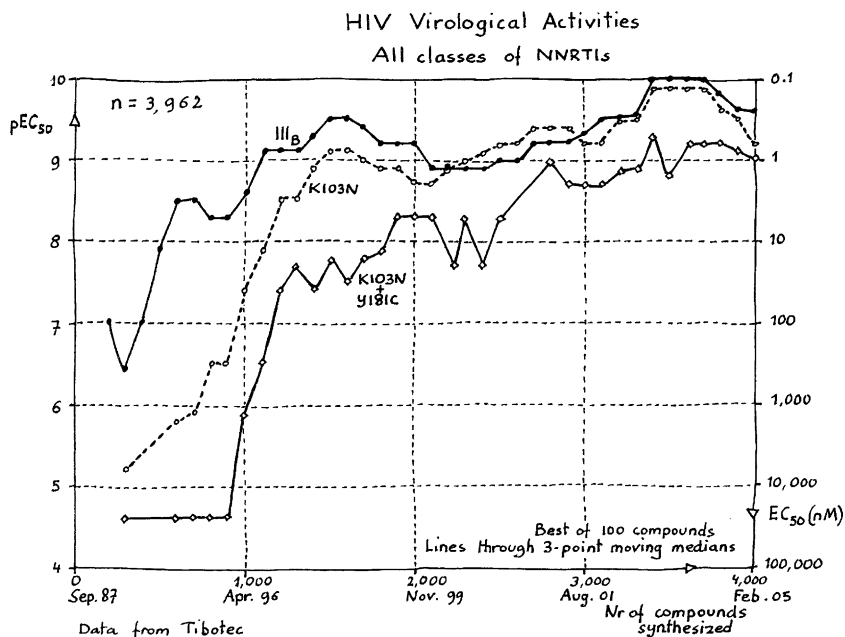
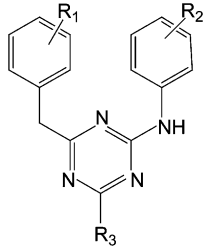


Figure 3. Evolution of the virological activity (pIC_{50}) obtained from the start of the NNRTI project in 1987 until February 2005. The vertical axis represents the median pIC_{50} of batches of consecutive 100 compounds that have been synthesized at Janssen Pharmaceutica and tested at the Rega Institute for Medical Research and Tibotec. A total of 3,962 compounds were synthesized during the period of 18 years. The three curves represent the evolution of activities against wild type virus (III_B), a representative single recombinant mutant (K103N) and a representative double mutant (K103N + Y181C).

To the date of writing about 4,000 compounds have been synthesized and tested virologically at Janssen and Tibotec in the search for the best possible NNRTI. Figure 3 illustrates the evolution over the duration of the project of the virological activity (pIC_{50}) of these compounds against wild type, single and double recombinant mutant viruses.

II. THE DIARYLTRIAZINE (DATA) SERIES

In an attempt to improve activity in the ITU-series it was decided to modify and substitute the thione-moiety with an imino-cyano group. Instead of obtaining the desired cyano-guanidino compound, a diamino-triazine derivative (**5a**, Table 1) was formed from the ring-closure of the intermediate cyano-guanidino derivative. This serendip-

Table 1. Activity (pIC₅₀) of tri-substituted triazines *vs* wt HIV-1 and selected single and double mutants^a


Compd	R ₁	R ₂	R ₃	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
5a	2,6-Cl ₂	4-CN	NH ₂	8.2	6.4	7.4	6.7	6.5	<5	5.9
5b	2-Cl	4-CN	NH ₂	7.9	5.6	6.4	5.6	5.2	nd	5.0
5c	H	4-CN	NH ₂	6.6	5.1	5.1	<4	nd	nd	nd
5d	2,6-Cl ₂	4-Cl	NH ₂	7.8	nd	6.5	5.5	5.1	nd	5.0
5e	2,6-Cl ₂	3-CN	NH ₂	6.6	5.1	5.6	5.1	nd	nd	5.1
5f	2,6-Cl ₂	4-CN	NHCH ₃	8.2	6.0	6.9	6.4	6.0	nd	5.3
5g	2,6-Cl ₂	4-CN	CONH ₂	8.6	6.5	7.5	6.6	6.6	nd	nd
5h	2,6-Cl ₂	4-CN	NHOH	8.6	7.0	7.9	7.6	7.6	5.3	6.5
5i	2,6-(Me) ₂	4-CN	NH ₂	8.9	5.9	7.4	6.7	7.0	nd	nd
5k	3,5-(Me) ₂	4-CN	NH ₂	7.1	<5	5.3	5.2	<5	nd	<4
5l	2,5-(Me) ₂	4-CN	NH ₂	8.5	5.2	6.6	5.8	6.1	nd	<4
5m	2,4-Cl ₂	4-CN	NH ₂	7.9	5.7	6.6	6.1	5.6	nd	5.3
5n	2,4,6-(Me) ₃	4-CN	NH ₂	9.1	7.1	8.5	7.8	7.3	5.3	7.3
5o	2,4,6-Cl ₃	4-CN	NH ₂	8.9	6.7	7.8	7.2	6.8	<5	nd
3	efavirenz			9.0	7.4	7.4	8.7	6.8	<5	7.4

^aThe LAI strain is the wild-type HIV-1 (wt, IIB). Recombinant viral strains with mutations in the RT enzyme are characterized according to the mutated amino acid position (one-letter codes). For example, K103N refers to replacement of lysine (K) at position 103 with asparagine (N). nd = not determined.

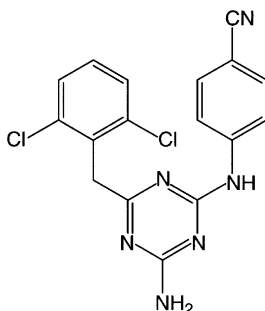
**5a**, R106168pIC₅₀ = 8.2

Figure 4. Structural formula of the prototype DATA compound and pIC₅₀ value for inhibition of wild-type HIV-1 replication.

itous discovery gave rise to the synthesis and design of a large number of DATA compounds with nano- and subnanomolar activity against wild-type (wt) virus and recombinant mutants derived from it. The best compounds in this series also showed high activity against clinical isolates of the virus.

Compound **5a**, the first analogue in the DATA series (Figure 4), displayed nanomolar activity against wt virus. Furthermore the compound was active against single recombinant mutants, such as L100I, Y181C, Y188L and particularly K103N. Micromolar activity was observed against the double mutant K103N + Y181C. These results gave rise to the development of a large synthetic program of DATA compounds.²⁰ The DATA molecule can be considered as built up from three parts consisting of the left wing, being the 2,6-dichlorophenyl part, the central triazine ring and the right wing, consisting of the 4-cyanophenyl part.

A. Substitutions at the right wing phenyl

From X-ray studies it was learned that the left part of the molecule is binding in the sub-pocket, which is formed by Y181, Y188, and W229.³ From subsequent molecular modelling it became clear that the cyano-moiety in the 4-position of the right phenyl undergoes a strong dipole-dipole interaction with the backbone carbonyl of H235. The NH-moiety, which connects the right phenyl with the central triazine ring

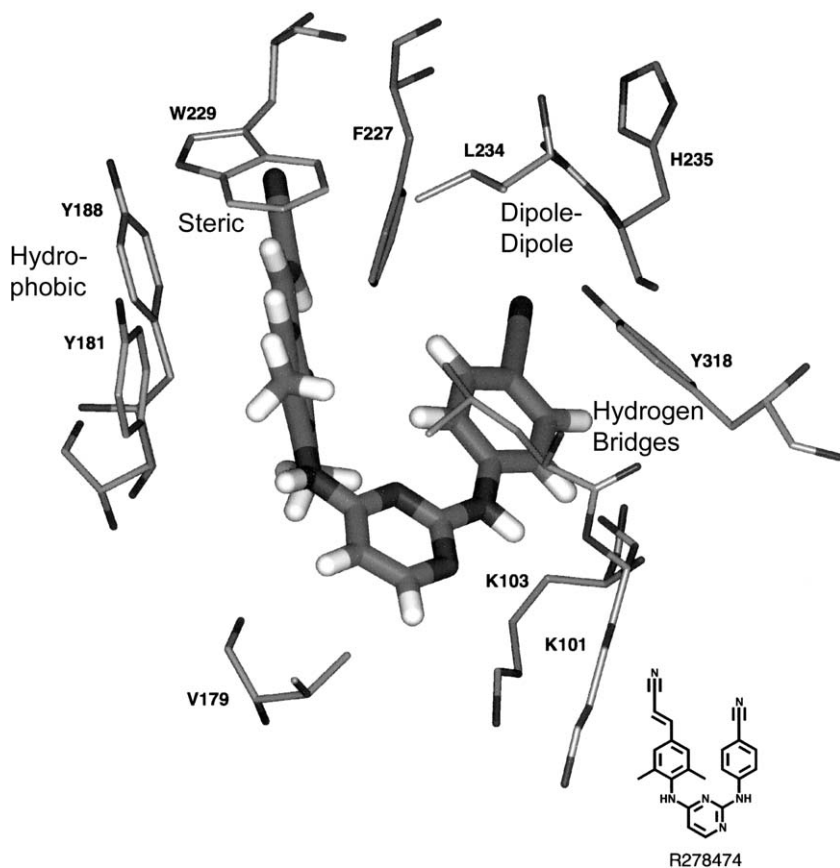


Figure 5. Molecular modelling of R278747 (TMC278) docked inside the non-nucleoside binding site of HIV-1 reverse transcriptase (RT), derived from the complex of R147681 (TMC120) with RT.² The various types of interaction of the ligand molecule with the residues and backbone of the protein are indicated.¹³

in the 2-position, forms a hydrogen bond with the backbone carbonyl of K101. The α -amino group of the same amino acid forms a hydrogen bond with the nitrogen of the triazine in the 1-position. Figure 5 illustrates the main molecular interactions between an NNRTI and the residues and backbone of the non-nucleoside binding site of the HIV-1 reverse transcriptase.¹³

A shift of the cyano-moiety from the 4- to the 3-position (**5e**, Table 1) demonstrated the superior activity of **5a** not only against the wt virus, but also against the single mutants and the double mutant K103N

+ Y181C. Introduction of a chlorine atom (**5d**) instead of the 4-cyano group gave a small difference in activity against wt virus which was in favour of **5a**. A more pronounced effect of more than one log unit difference was observed with this compound against the single mutants Y181C and Y188L. These small chemical modifications showed that the cyano group in the 4-position was crucial in that part of the molecule for high activity against both wild type and mutant virus.

B. Substitutions at the left wing phenyl

Regio-substituent modifications were performed on the left wing phenyl ring of the molecule. The activity of the un-substituted phenyl analogue (**5c**) indicated that the presence of chlorines in the 2,6-position was necessary for acceptable activity against wt virus and the single mutants L100I, K103N and Y181C. The mono-substituted 2-chlorine analogue (**5b**) displayed a stronger activity than **5c** against wt virus. The increase of activity was confirmed against the single mutants. Replacement of the chlorines in the 2,6-position by methyl groups (**5i**) clearly improved the activity against wt virus. Activity decreased against L100I and was equal against K103N and Y181C, but increased against Y188L.

Shifting the methyl group in the 6-position to the 5-position (**5l**) gave a small effect on the activity against wt virus, but there was approximately one log unit difference against the single mutants K103N, Y181C and Y188L in favour of **5i**. A comparison of the 2,6- (**5i**) and the 3,5-dimethyl (**5k**) analogues made clear that the activity of the former exceeded that of the latter with more than one log unit against wt virus and the four single mutants within the panel. An encouraging activity was found in the 2,4-dichloro derivative (**5m**) against all the tested viruses.

From these results it was decided to synthesize the 2,4,6-trichloro compound (**5o**). Superior activity was noted against wt virus and single mutants, and significant progress was made against Y181C. Substitution of the chlorine atoms by methyl moieties (**5n**) resulted in another increase of activity, which was comparable to that of efavirenz against wt virus and the double mutant K103N + Y181C (Figure 1, Table 1). Compound **5n** was clearly superior to efavirenz with regard to the single mutants K103N and Y188L, but efavirenz was still superior against the single mutants L100I and Y181C.

C. Substitutions at the 6-position of triazine

The 6-amino substituent in the central triazine ring of compound **5a** was replaced with several other moieties. Mono-methylation of the 6-amino group (**5f**) retained activity against wt virus, but results from all single mutants within the panel clearly demonstrated the higher activity of **5a** (Table 1).

Introduction of a primary amide group in the 6-position of the triazine ring **5g** had a favorable effect on activity, creating a compound that was superior to **5a** with regard to wt virus. Compound **5h** (R3 = NHOH) was clearly more potent than **5a** against wt virus, the four single mutants, and also against the double mutant K103N + Y188L.

D. Variation of the linker between the left wing and triazine

With compound **5a** as basic structure, it appeared that the CH₂-linker, which connects the central triazine ring and the left 2,6-dichlorophenyl ring, could easily be replaced with several other linkers (**6a**, **6b**, **6c**, Table 2). Substitution of the CH₂-linker with an NH group (**6a**) gave an increase in activity against wt virus and against the single mutants L100I and Y181C. It had no effect on K103N, but de-

Table 2. Activity (pIC₅₀) of tri-substituted triazines *vs* wt HIV-1 and selected single and double mutants

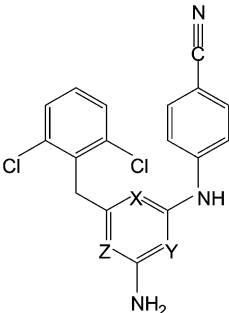
Compd	X	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
5a	CH ₂	8.2	6.4	7.4	6.7	6.5	<5	5.9
6a	NH	8.5	6.6	7.4	7.0	5.7	nd	5.8
6b	O	8.5	6.4	7.1	6.6	5.5	nd	5.5
6c	S	8.5	6.8	7.0	6.6	6.1	nd	nd

creased activity against Y188L. Compounds **6b** (X=O) and **6c** (X=S) were equivalent to **6a** and slightly superior to **5a** against wt virus. On the other hand, both compounds (**6b**, **6c**) were less active against the single mutants K103N and Y188L. The sulphur analogue **6c** was more potent than **5a** against the single mutant L100I. In contrast, **6b** was equipotent to **5a** against this single mutant. Both compounds (**6b**, **6c**) and **5a** displayed similar activity to the Y181C mutant.¹⁹ Compared to **5a**, compound **6a** in general demonstrated equivalent or superior activity against the majority of the viruses tested within the panel, except for the mutant Y188L, where **5a** was more potent.

III. THE DIARYLPYRIMIDINE (DAPY) SERIES

Replacement of the triazine central ring in the DATAs with a pyrimidine ring can generate three isomeric pyrimidines by replacing any one nitrogen atom in the triazine ring with a carbon atom.²¹ Preparation of all three regio-isomers **7a–c** revealed that compound **7a** displayed the highest anti-HIV activity, superior to **5a** against wt virus and the single mutants K103N and Y188L (Table 3). The activity was similar for the single mutants L100I and Y181C. Molecular modelling studies had predicted this outcome. The compound forms two hydrogen bonds

Table 3. Activity (pIC₅₀) of isomeric pyrimidines *vs* wt HIV-1 and selected single and double mutants

										
Compd	X	Y	Z	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
7a	N	N	CH	9.1	6.5	7.9	6.8	7.1	5.1	6.2
7b	N	CH	N	6.3	<4	<4	<4	<4	nd	<4
7c	CH	N	N	8.0	<4	4.5	<4	<4	nd	nd

with K101. The NH group, which connects the central pyrimidine ring with the 4-cyano-phenyl moiety, is responsible for a strong hydrogen bond interaction with the main-chain carbonyl oxygen of Lys 101. The pyrimidine nitrogen in the 1-position serves as hydrogen bond acceptor of the α -amino group of K101 (Figure 5). Furthermore, the compound gave the best docking results of all three possible regio-isomers. In general, there was a small difference in wild type activity between the triazines (DATAs) and the pyrimidines (DAPYs) in favor of the DAPYs.

The question was raised whether it was possible to simplify the chemical structure of the molecules by omission of the amino group in the 6-position of the central pyrimidine ring. Molecular modelling studies done on these proposed structures indicated that the amino part of the molecule did only play a minor role in the binding process. The virological activities of the synthesized compounds **8h** and **8j** (Table 4) confirmed the modelling results. This encouraged synthesizing a large series of DAPY compounds. The compounds with the highest activity displayed a 2,4,6-trisubstituted pattern on the left wing phenyl ring. Within this series, compounds **8n** and **8m** appeared to be the most interesting, as they displayed high activity against wt virus, comparable to that of efavirenz (Table 4). Activities of these compounds against the single mutants L100I, Y181C and the double mutant K103N + Y181C were of the same order of magnitude as those of efavirenz. This is in contrast with the mutants K103N and Y188L, where both compounds (**8n**, **8m**) were clearly superior to efavirenz.

Variation of the linker between the left wing phenyl and pyrimidine from NH to oxygen (**8a** and **8f**) or sulphur (**8g**) confirmed the superior activity of **8m** and **8n**. Methylation of the NH-linker (**8l**) produced a similar activity against wild-type virus as with **8a**.

Replacement of the 4-methyl moiety (**8n**) with chlorine (**8o**) corroborated the high activity of **8n**. Replacement of the 2,6-dimethyl group (**8n**) with a 2,6-dibromo moiety (**8s**) enhanced activity against wt virus and three single mutants L100I, K103N and Y181C. Compound **8n** (R147681, TMC120) was developed further, and received the generic name dapivirine. It is regarded as the prototype DAPY compound (Figure 6). This compound is being developed as a microbicide for prevention of HIV infection.^{5,24}

Introduction of a chlorine atom (**9b**, Table 5) or a carboxamide moiety (**9a**) for the 4-cyano group (**8n**) made clear that the presence of a cyano group was necessary for high activity against wt virus, the four single mutants and the double mutant K103N + Y181C.

It is somewhat surprising that highly lipophilic compounds (with $\log P > 5$), such as dapivirine and its analogues, often show good oral

Table 4. Activity (pIC₅₀) of 2,4-disubstituted pyrimidines *vs* wt HIV-1 and selected single and double mutants

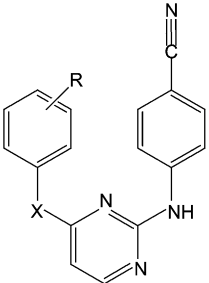
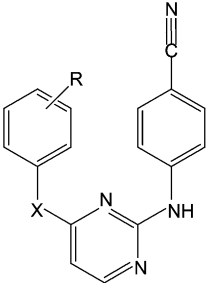
									
Compd	R	X	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
8a	2,4,6-(CH ₃) ₃	O	8.4	7.7	8.5	7.4	7.3	5.1	6
8b	2,4-Cl ₂ ,6-CH ₃	O	7.0	5.1	6.3	5.1	5.0	nd	nd
8c	2,4,6-Cl ₃	O	6.5	4.9	5.5	4.1	<4	nd	nd
8d	2,6-(CH ₃) ₂ ,4-Br	O	8.6	7.0	8.3	7.3	7.1	5.7	6.7
8e	2-Br,4-Cl,6-CH ₃	O	8.0	6.5	7.3	6.3	6.1	nd	nd
8f	2,6-(CH ₃) ₂ ,4-CN	O	9.0	7.1	8.6	7.5	7.7	6.1	7.0
8g	2,4,6-(CH ₃) ₃	S	8.5	7.3	8.0	7.6	7.7	5.3	nd
8h	2,6-Cl ₂	S	8.9	6.9	6.9	6.5	6.7	nd	nd

Table 4. — Continued

									
Compd	R	X	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
8i	2,6-(CH ₃) ₂ ,3-NH ₂	NH	8.6	6.1	7.2	6.2	<5	nd	nd
8j	2,6-Cl ₂	NH	7.5	<4	<4	<4	<4	nd	nd
8k	2-Br,4-F,6-Cl	NH	8.7	<4	7.5	7.2	<4	nd	nd
8l	2,4,6-(CH ₃) ₃	NCH ₃	8.2	6.9	7.1	7.0	6.4	nd	nd
8m	2,6-(CH ₃) ₂ ,4-CN	NH	9.4	7.5	8.7	8.2	8.1	6.0	7.5
8n	2,4,6-(CH ₃) ₃	NH	9.0	7.8	8.4	8.1	7.4	<5	7.4
8o	2,6-(CH ₃) ₂ ,4-Cl	NH	8.7	6.7	7.9	7.7	7.0	<5	6.4
8p	2,6-Cl ₂ ,4-CN	NH	7.2	nd	nd	nd	nd	nd	nd
8q	2,4-Cl ₂ ,6-CH ₃	NH	9.0	7.2	7.9	7.8	7.1	<5	nd
8r	2,4-Br ₂ ,3,6-Cl ₂	NH	8.6	7.3	7.8	7.2	7.0	nd	nd
8s	2,6-Br ₂ ,4-CH ₃	NH	9.2	8.5	8.5	8.5	<4	<4	<4
efavirenz			9.0	7.4	7.4	8.7	6.8	<5	7.4

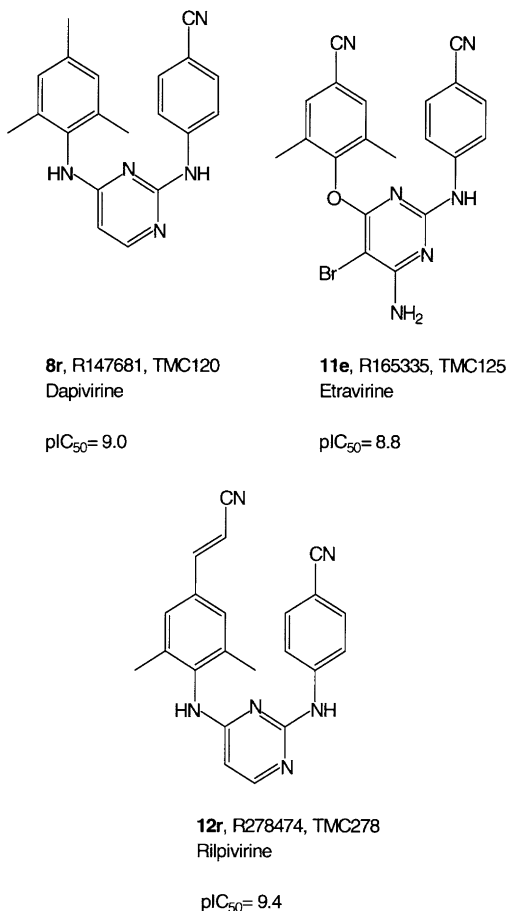
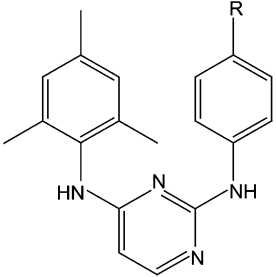


Figure 6. Structural formulas of three DAPY compounds that are in advanced stages of clinical investigation at the time of writing: R147681 (TMC120, dapivirine). R165335 (TMC125, etravirine) and R278474 (TMC278, rilpivirine), with their respective pIC_{50} values for inhibition of wild-type HIV-1 replication.

bioavailability.¹⁸ Paul Janssen had hypothesized that this could be due to the spontaneous formation of aggregates by these compounds and subsequent transcytosis through epithelial or other specialized cells in the gastro-intestinal tract.¹³ It was confirmed by means of dynamic light scattering (DLS) that aggregates of a theoretically predicted diameter of about 100 nm are indeed formed by many of the DAPY analogues under the conditions of pH and concentration that prevail in the gastro-intestinal tract.²⁵ These aggregates can also be

Table 5. Activity (pIC₅₀) of 2,4-disubstituted pyrimidines *vs* wt HIV-1 and selected single and double mutants

								
Compd	R	LAI	L100I	K103N	Y181C	Y188L	Y100I + K103N	K103N + 181C
9a	CONH ₂	7.2	<5	<5	<5	<5	nd	nd
9b	Cl	8.5	6.7	7.6	7.1	5.6	<5	nd
8n	CN	9.0	7.8	8.4	8.1	7.4	<5	7.4

observed directly by means of electron microscopy²⁶ and atomic force microscopy,¹² such as is illustrated by [Figure 7](#).

A. Substitutions at the 5-position of pyrimidine

In contrast with the diaryltriazines (DATAs), it was possible within the DAPY series to evaluate the effects on virological activity of different substituents in the 5-position of the central pyrimidine ring ([Table 6](#)). Introduction of bromine in position five of the central pyrimidine ring of dapivirine (**10m**, Y=Br, R=CH₃) gave no improvement in activity against wt virus, the single mutants and the double mutant K103N + Y181C. A marked improvement was seen, however, against the double mutant L100I + K103N. Substitution of bromine with the smaller chlorine atom **10o** (Y=Cl, R=CH₃) gave minor differences in activity. The analogue compound with an acetylene moiety in the 5-position of the pyrimidine ring (**10p**) yielded similar activities as compounds **10m** and **10o** against wt virus and the single mutants. The activity was improved significantly, however, against the double mutants L100I + K103N and K103N + Y181C. The cyano-isoster **10n** was more potent against wt virus than **10p**, but this result was not obtained against the single mutants, with the exception of K103N.

Table 6. Activity (pIC₅₀) of 2,4,5-trisubstituted pyrimidines *vs* wt HIV-1 and selected single and double mutants

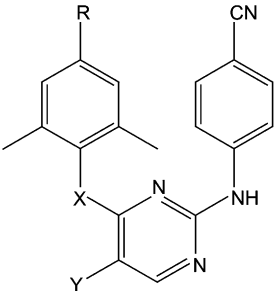
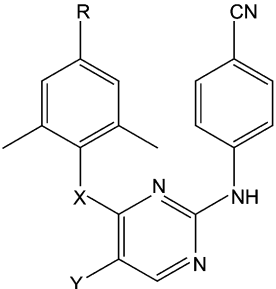
										
Compd	R	X	Y	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
10a	CN	O	Br	8.9	8.2	8.9	7.7	8.2	7.3	7.6
10b	CH ₃	O	Cl	8.0	7.4	7.9	7.1	7.5	nd	nd
10c	CH ₃	O	Br	8.0	7.7	8.1	7.2	7.5	6.6	7.0
10d	CN	O	NH ₂	9.1	7.9	8.9	8.0	8.3	6.6	7.9
10e	Cl	O	Cl	8.0	7.2	7.8	7.0	7.8	nd	nd
10f	CN	O	NHCOCH ₃	8.6	7.5	8.5	7.6	8.1	6.7	7.6
10g	CN	O	NO ₂	7.5	6.9	8.0	6.5	6.1	<5	6.7
10h	Br	O	Cl	8.1	7.1	7.7	6.9	7.2	5.9	6.2
10i	CN	O	Cl	8.9	8.1	8.6	7.5	8.4	6.9	8.2
10j	CH ₃	S	Cl	6.9	5.9	6.6	6.0	6.4	nd	nd

Table 6. — *Continued*

										
Compd	R	X	Y	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
10k	CN	NH	Br	9.5	8.2	9.4	8.0	8.5	7.4	7.5
10l	CH ₃	NH	CH ₃	8.8	7.9	8.6	7.9	7.7	<5	7.3
10m	CH ₃	NH	Br	8.3	8.0	8.1	7.4	7.6	6.5	6.6
10n	CH ₃	NH	CN	9.0	7.2	8.5	7.4	7.2	nd	nd
10o	CH ₃	NH	Cl	8.6	7.8	8.2	7.5	7.5	6.5	6.9
10p	CH ₃	NH	CCH	8.4	7.6	8.2	7.4	7.4	7.2	7.4
10q	Br	NH	CN	8.7	6.9	8.2	7.5	7.4	5.9	6.6
10r	CN	NH	Cl	8.9	8.3	9.1	7.8	8.3	7.0	7.2
10s	Br	NH	Br	8.6	7.9	8.5	7.3	7.9	6.4	nd
10t	CN	NH	CH ₃	9.2	7.3	8.5	7.4	7.6	<6	nd
10u	Br	NH	Cl	8.6	7.5	8.4	7.4	7.7	<5	6.8

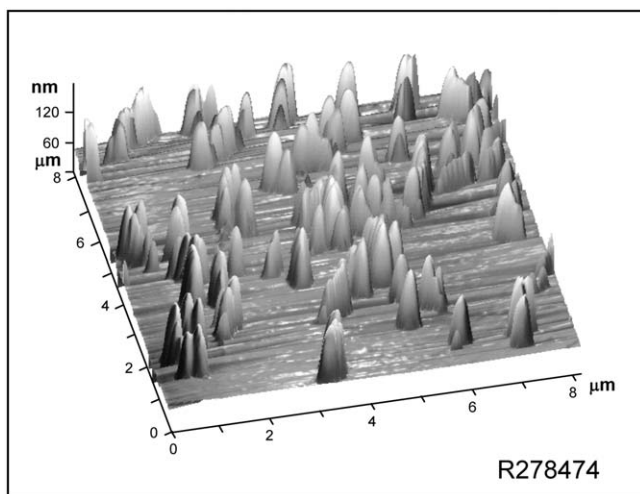
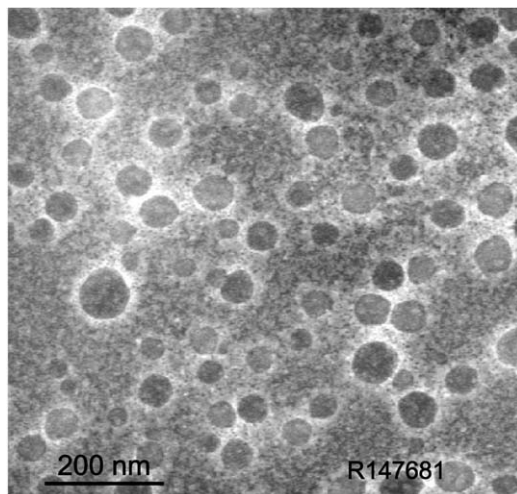


Figure 7. Aggregates of about 100 nm diameter formed by DAPY compounds, as observed by electron microscopy (R147681, TMC120)²⁶ and by atomic force microscopy (R278474, TMC278).¹²

B. Substitutions at the 4-position of the left wing phenyl

It was of interest to evaluate the effects on virological activity of substituent variation in the 4-position of the left wing phenyl ring and to study the effect of the linker connecting this ring with the cen-

tral pyrimidine ring. Substitution of the methyl moiety in **10n** with bromine (**10q**) generally led to a diminution in activity against wt virus and the single mutants L100I and K103N (Table 6). On the other hand, a small increase in activity was seen against the single mutants Y181C and Y188L. A comparison of compound **10m** (Y=Br, R=CH₃) with **10s** (Y=R=Br) showed an increase of activity against wt virus and the single mutants K103N, Y188L, while a similar activity was observed against the single mutants L100I and Y181C. Great improvement was obtained with the introduction of a CN group (**10k**) in the place of the methyl moiety (**10m**) in the 4-position of the left wing phenyl group. The activity was not only greatly increased against wt virus but also against all the single mutants and the double mutants within the panel. This result was confirmed by comparison of compound **10k** with compounds **10o** and **10r**.

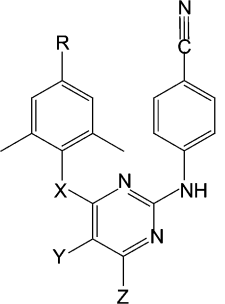
C. Variations of the linker between left wing and pyrimidine

A comparison of compound **10a**, in which the NH-linker was replaced with oxygen, with the parent compound (**10k**) showed that the activity against wt virus, the single mutants K103N, Y181C, Y188L was higher for **10k** and differed slightly against the double mutant L100I + K103N (Table 6). Similar activity was observed against the single mutant L100I, and a slight increase was noted against the double mutant K103N + Y181C. The differences in activity of compounds **10i** and **10r** are small against all viruses within the panel, except for K103N where the difference is in favor of **10r**. This contrasts with the better results that were obtained with **10i** against the double mutant K103N + Y181C. Altogether, there was not much difference in activity between the NH and the oxygen linkers. Introduction of a sulphur linker (**10j**) instead of an NH linker (**10o**) demonstrated the superior activity of **10o** against wt virus and all the single mutants within the panel.

D. Substitutions at the 5- and 6-positions of pyrimidine

The presence of the central pyrimidine ring allowed generating tetra-substituted pyrimidine analogues with the potential to increase activity against double mutants (Table 7). The introduction of an amino-moiety in the 6-position of the pyrimidine ring of **10o** yielded compound **11a**, which resulted in increased activity. The progress was marginal for wt virus and the single mutant Y181C, but improvements of a half log unit or more were made with the single mutants L100I,

Table 7. Activity (pIC₅₀) of 2,4,5,6-tetrasubstituted pyrimidines *vs* wt HIV-1 and selected single and double mutants

											
Compd	R	X	Y	Z	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
11a	CH ₃	NH	Cl	NH ₂	8.7	8.1	8.8	7.6	8.0	7.3	7.5
11b	CN	NH	Cl	NH ₂	9.2	8.2	9.4	8.0	8.3	7.6	7.8
11c	CH ₃	O	Cl	NH ₂	8.0	7.1	7.9	6.8	7.2	6.6	7.0
11d	CN	O	Cl	NH ₂	8.9	8.1	8.8	7.8	8.2	7.5	8.1
11e	CN	O	Br	NH ₂	8.8	8.5	8.9	8.2	8.6	7.8	8.3
11f	CH ₃	O	Br	NH ₂	7.9	7.1	7.5	6.5	6.7	6.7	7.0
11g	CH ₃	O	Br	NHCH ₃	7.8	nd	nd	nd	nd	nd	nd
11h	CN	O	Br	NHCH ₃	8.4	8.2	8.3	7.6	8.0	7.5	7.9
11i	CN	O	Br	CH ₃	8.4	8.2	8.2	7.5	8.1	7.5	7.8
11j	CN	O	Br	CH ₂ OH	8.4	8.7	8.4	8.2	8.2	8.2	8.3
11k	N(CH ₃) ₂	O	Br	NH ₂	7.9	7.5	8.1	7.5	7.6	nd	nd
11l	CH ₃	NH	Br	CONH ₂	8.5	9.0	9.1	7.7	7.9	nd	nd

K103N, Y188L and particularly with both double mutants L100I + K103N and K103N + Y181C.

Substitution of the 4-methyl group on the left wing phenyl ring in **11a** ($R = CH_3$) with a CN moiety (**11b**) gave a further increase in activity against all the viruses within the panel. There was superior activity with this compound against the single mutant K103N. Changing the NH linker, which connects the central pyrimidine ring with the left wing phenyl ring, into an oxygen atom (**11d**) was in favor of **11b** with the exception of the double mutant K103N + Y181C.

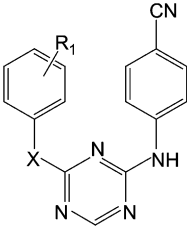
Substitution of the chlorine atom in the 5-position of the pyrimidine ring with a bromine atom (**11e**) had a marginal effect on activity against wt virus, which was in contrast with the superior activity against the single and double mutants. Compound **11e** (R165335, TMC125) is in clinical development under the generic name etravirine.^{1,4} Etravirine has been studied in treatment-naïve patients infected with HIV-1 and produced a significant decrease in viral load with respect to untreated patients after one week of oral mono-therapy.¹⁰ Treatment of patients harboring resistant HIV-1 virus against at least one of the currently marketed NNRTIs also gave favorable results in oral mono-therapy after one week of treatment.⁸

Demethylation of the amino-moiety (**11h**) in the 6-position of the pyrimidine ring (**11e**) had a favorable effect on activity against all viruses within the panel. Replacement of the methyl-amino-group with a methyl moiety (**11i**) had virtually no effect on activity (Table 7). The carbinol analogue (**11j**) derived from (**11i**) displayed a lower activity against wt virus and the single mutants K103N and Y188L than **11e**, but it was superior to the double mutant L100I + K103N. Modifications of the cyano group on the left wing phenyl ring by a dimethyl-amino (**11k**), or methyl moiety (**11f**) clearly showed that a cyano group in that position can be associated with high activity. Replacement of the primary amino group in **11f** with a primary amide moiety **11l** and switching the linker from oxygen to a secondary amino group led to a significant improvement of activity not only against wt virus but also against all single mutants.

E. Replacement of the central pyrimidine by triazine

The next step in the research on NNRTIs was to replace the central pyrimidine ring with a symmetric triazine ring, giving rise to the synthesis of compounds **12a-j** (Table 8). In a sense, this constituted a temporary return from the current DAPY design to the preceding DATA concept. The isosteric analogue (**12e**) of dapivirine (**8n**) yielded

Table 8. Activity (pIC₅₀) of bi-substituted triazines *vs* wt HIV-1 and selected single and double mutants

									
Compd	R ₁	X	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
12a	2,4,6-(CH ₃) ₃	O	9.2	7.7	8.5	7.7	7.2	<5	nd
12b	2,6-Br ₂ ,4-CH ₃	O	8.9	7.8	8.2	7.4	6.4	6.8	6.8
12c	2,4,6-(CH ₃) ₃	S	8.7	7.5	8.5	7.9	7.7	<5	nd
12d	2-Cl,4-Br,6-CH ₃	O	9.0	7.1	7.9	7.2	6.6	<5	nd
12e	2,4,6-(CH ₃) ₃	NH	9.5	7.9	8.5	8.2	7.4	5.9	7.3
12f	2,6-Br ₂ ,4-CH ₃	NH	9.3	8.5	8.6	8.5	7.1	<5	nd
12g	2,6-(CH ₃) ₂ ,4-CN	NH	9.1	6.6	8.1	7.3	7.3	5.6	6.9
12h	2,6-(CH ₃) ₂ ,4-Br	NH	9.2	7.1	8.2	7.8	7.2	5.5	6.9
12i	2,6-(CH ₃) ₂ ,4-CN	O	8.4	6.0	7.7	6.3	6.6	5.4	6.4
12j	2,6-(CH ₃) ₂ ,4-t-Bu	NH	8.6	6.5	7.5	7.1	5.7	nd	nd

an improvement of antiviral activity against wt virus, the single mutants and the double mutant L100I + K103N. The activity of the compound against the other double mutant K103N + Y181C remained similar.

Compounds with an NH linker (**12e**, **12g**) between the central triazine ring and the left wing phenyl ring yielded superior activity against wild-type when compared to the corresponding compounds with oxygen (**12a**, **12j**) and sulfur linker (**12c**). The mother compound (**12e**) was more potent than the oxygen analogue (**12a**) against Y188L. The analogue with the sulfur linker (**12c**) was more potent than both the oxygen analogue (**12a**) and the mother compound (**12e**) against the same mutant Y188L.

The methyl substituent in the 4-position on the left wing phenyl ring could be replaced with a bromine atom or another voluminous group, such as a tertiary butyl moiety (**12j**), without a loss of more than two log units.

F. Variations on the 4-cyano left wing phenyl

The question was raised which modifications can be applied in the 4-position on the 2,6-dimethyl-substituted-phenyl in order to improve virological activity. The problem was studied on the pyrimidine series with compound **8m** as a starting point. Incorporation of a spacer consisting of carbon atoms, or a vinyl group between the CN moiety and the left wing phenyl ring gave rise to a series of compounds presented in Table 9.

Chain elongation with two carbons to compound **13c** resulted in a remarkable improvement with more than one log unit of activity, in particular against all single mutants within the panel. Compared to compound **8m**, a superior activity against both double mutants was observed. Insertion of a vinyl group between the CN moiety and the phenyl ring yielded compound **13k** with E-geometry.^{11,13} Activity of this compound against wt-virus was comparable to that of **8m**, whereas the activity against the single and double mutants in the panel was markedly improved.

A comparison of the activity spectrum of **13k** with that of efavirenz shows that **13k** possesses superior activity against all viruses within the panel, with a difference of approximately two log units against the single mutants L100I, K103N, Y188L and the two double mutants. The corresponding Z-isomer **13i** displayed a weaker activity against all viruses within the panel compared to the E-enantiomer, but it was still better than efavirenz. Compound **13k** (R278474, TMC278) has re-

Table 9. Activity (pIC₅₀) of 2,4-disubstituted pyrimidines *vs* wt HIV-1 and selected single and double mutants

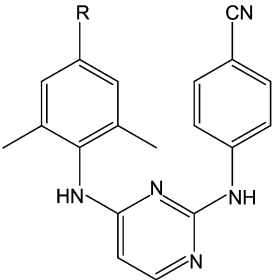
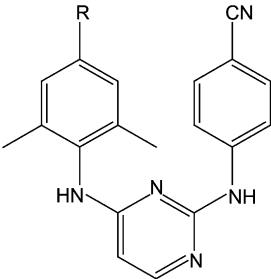
								
Compd	R	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
13a	C(CH ₃) ₃	8.0	6.9	7.3	7.1	5.7	nd	nd
13b	CH ₂ CN	9.1	7.4	8.1	7.6	6.6	nd	nd
13c	CH ₂ CH ₂ CN	9.2	9.1	9.4	8.9	7.7	7.5	8.6
13d	COCH ₂ CN	7.6	6.5	7.3	6.5	6.1	5.4	nd
13e	CHCHCH ₂ CN	9.0	7.4	8.6	7.5	7.7	6.6	nd
13f	CHCBr ₂	8.2	7.5	8.1	7.4	7.4	5.9	nd
13g	CHC(CH ₃)CN(E)	9.2	9.5	9.5	8.9	8.2	7.4	8.4
13h	CHC(CH ₃)CN(Z)	9.1	8.5	9.0	8.4	7.5	<5	7.7
13i	CHCHCN(Z)	9.2	8.2	8.8	8.3	7.5	6.1	7.4

Table 9. — *Continued*

								
Compd	R	LAI	L100I	K103N	Y181C	Y188L	L100I + K103N	K103N + Y181C
13j	C(CH ₃)CHCN(E)	9.0	9.1	9.0	8.6	8.4	7.6	8.6
13k	CHCHCN(E)	9.4	9.4	9.5	8.9	8.7	8.1	9.3
13l	C ₆ H ₅	7.9	6.7	7.8	6.7	6.8	nd	nd
13m	CH ₂ O(CH ₂) ₂ CN	9.2	7.6	8.5	7.5	7.4	nd	nd
13n	CClCHCN	8.4	8.6	8.7	8.2	8.1	7.5	8.2
13o	CHC(CH ₂ CN)CN	9.4	7.5	8.2	8.2	7.5	nd	nd
13p	CH ₂ OCH ₂ CH ₃	8.4	6.7	7.5	6.7	6.9	<5	nd
efavirenz		9.0	7.4	7.4	8.7	6.8	<5	7.4

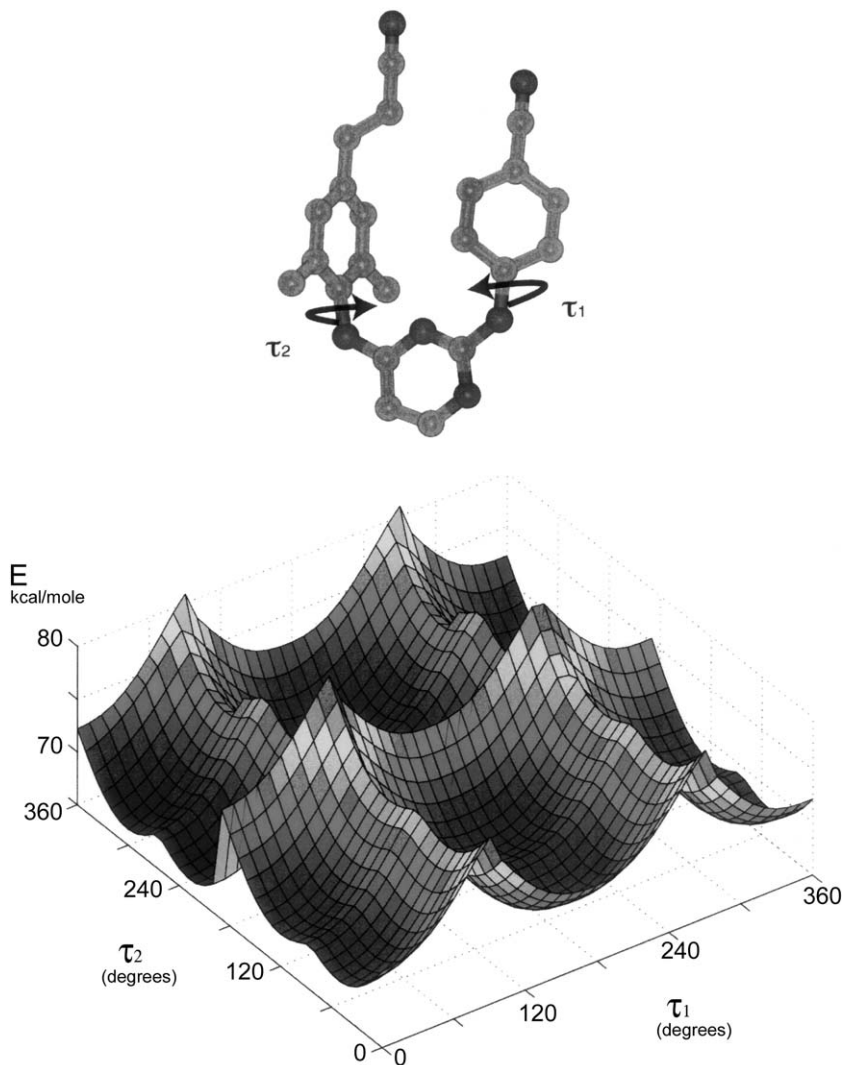


Figure 8. Torsional flexibility of a DAPY compound (R278474, TMC278) produced by the rotations about the linkers that join the left and right wing phenyl rings with the central pyrimidine moiety (left panel) and the corresponding enthalpy change surface as a function of the two torsion angles (right panel).¹³

ceived the generic name of rilpivirine. It is in an advanced stage of clinical investigation for oral treatment against HIV-1 infections in patients.⁹

Introduction of a methyl group in **13k** in position alpha or beta with regard to the CN moiety produced compounds **13g** and **13j**, respectively, both showing high activity against all viruses within the panel. The cyano-vinyl parent compound (**13k**) was also superior in activity against all viruses in the panel in comparison to its dibromo-vinyl analogue (**13f**).

While the DAPY compounds are often described as “tight binding” it is also thought that their high barrier against resistance formation (or resilience) is due to the great flexibility of their chemical structure. This may result in multiple binding modes that can compensate for changes in the geometry of a mutated binding site.^{17,3} The torsional flexibility of DAPY compounds is illustrated by [Figure 8](#), which shows the effect of torsional rotations about the linkers that join the left and right wing phenyls to the central pyrimidine.

IV. CONCLUSION

Dr. Paul Janssen had a dream, when he founded his research laboratory, that medicinal chemistry could relieve the suffering of mankind in areas of important diseases. In this respect he has been highly successful. Sometimes success came quickly, as in the case of analgesics, neuroleptics, anthelmintics, antimycotics and gastro-intestinal compounds. In his fight against HIV, however, progress has been laborious, costly and time-consuming, but, in the end, the effort proved to be worthwhile.

ACKNOWLEDGEMENTS

With this contribution the authors wish to pay tribute to a great man and to a great company that made the scientific Odyssey of the DATA and DAPY HIV-1 NNRTIs possible. Without the late Dr. Paul Janssen and his many close and distant collaborators this historical account would not have been written.

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