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Perspective

Toward Improved Anti-HIV Chemotherapy: Therapeutic Strategies for Intervention with HIV Infections

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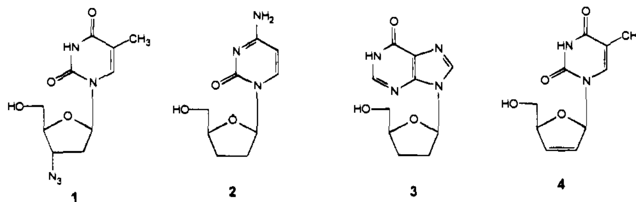
Introduction

The anti-HIV chemotherapy era started a decade ago when suramin was found to protect human T lymphocytes against the infectivity and cytopathicity of human immunodeficiency virus (HIV).[1] This finding shortly followed the successful isolation of HIV in cell culture[2,3] and was guided by earlier observations[4] on the inhibitory effects of suramin on the reverse transcriptase activity of murine and avian retroviruses.[5] Suramin,[6] as well as the polyoxometalate HPA 23,[7] were also the first antiviral agents shown to inhibit HIV replication *in vivo*, albeit in a limited number of AIDS patients. However, suramin was discontinued as a therapeutic modality for the treatment of HIV infections after additional short-term clinical studies showed it to be too toxic and of little, if any, clinical benefit.[8]

Meanwhile,[9] azidothymidine (AZT, zidovudine) (1) had been discovered to inhibit the infectivity and cytopathicity of HIV at much lower concentrations than suramin. After initial clinical studies showed it to offer some clinical as well as immunological benefit,[10] AZT was submitted to a double-blind, placebo-controlled trial, which established its efficacy in decreasing mortality and reducing the frequency of opportunistic infections, at least over a 24-week observation period.[11] Admittedly, adverse reactions, particularly bone marrow suppression (i.e. anemia and neutropenia) were also observed,[12] but these common complications of AZT treatment were considered to be outweighed by the clinical benefit achieved. Follow-up studies then confirmed that prolonged AZT therapy delayed progression of the disease in patients with AIDS,[13,14] in patients with symptomatic HIV infection,[15,16] and in patients with asymptomatic HIV infection.[17,18] These beneficial effects were sustained upon reducing the daily dosage regimen (from 1500 mg to 600 mg or even 300 mg),[19,20] and they were reflected by an improvement in survival of the patients with AIDS,[21] and, as with the adults, children with symptomatic HIV infection were also found to benefit from AZT treatment.[22,23]

Shortly after AZT, two other 2′,3′-dideoxynucleosides, *viz.* 2′,3′-dideoxycytidine (DDC, zalcitabine) (**2**) and 2′,3′-dideoxyinosine (DDI, didanosine) (**3**) were reported to inhibit the infectivity and cytopathicity of HIV.[24] DDC and DDI showed benefit in the treatment of HIV infections, first in preliminary clinical trials[25,26] and subsequently in more extended clinical trials in both adults[27-30] and children.[31] DDC and DDI, like AZT, were then approved for clinical use in the treatment of HIV infections, albeit with more restrictions than for AZT, i.e. DDC only in combination with AZT, and DDI only for those patients that had developed resistance or intolerance to AZT. In addition to AZT (Retrovir), DDC (Hivid) and DDI (Videx), other 2′,3′-dideoxynucleoside analogues such as 2′,3′-didehydro-2′,3′-dideoxythymidine (D4T, stavudine) (**4**) have also entered clinical trials,[32] and D4T (Zerit) has been recently approved for clinical use.



The initial successes obtained with the 2′,3′-dideoxynucleoside analogues in the treatment of HIV infections,

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both *in vitro* and *in vivo*, have prompted the search for other HIV inhibitors, some targeted at the reverse transcriptase (RT) and others targeted at viral processes other than RT. We now have at hand a variety of compounds belonging to the classes of the polyanionic substances, bicyclam derivatives, dideoxynucleoside analogues, acyclic nucleoside phosphonates, non-nucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors that appear to be excellent candidates for further clinical development. Some of the bicyclams, NNRTIs, and protease inhibitors inhibit HIV replication in cell culture at nanomolar concentrations without being toxic to the host cells at concentrations up to 1 mM, thus achieving *in vitro* selectivity indexes of 100 000 and higher. Such selectivity has been rarely achieved in the antiviral chemotherapy field.

Yet, the initial, unjustified expectations that a "cure" would be rapidly found for AIDS have waned, for a number of reasons. None of the anti-HIV drugs that have been formally approved, or will shortly be approved, for the treatment of HIV infections have proved capable of eradicating the infection. Although they suppress virus replication, at the current dosage regimens used they do not prevent drug-resistant virus strains from arising. Although it is not clear to what extent these drug-resistant strains are pathogenic, it is generally assumed that their emergence impedes the recovery process and contributes to the progression of the disease. It is obvious that anti-HIV therapy should not be delayed until the disease is too advanced, but how early postinfection it has to be installed has remained a matter of conjecture. Ideally, the appropriate anti-HIV drug(s), or combination thereof, should be given as soon as it has become evident that the virus is replicating, and the dosage should be such that virus replication is shut off completely so as to avoid resistant strains from emerging.

Although immunodeficiency is the hallmark of AIDS, this is essentially a viral disease, and thus should be treated, in the first place, by antiviral agents. It is now apparent that from the early stage of infection many more cells are infected throughout the lymphoid system than had initially been estimated based on examination of $CD4^+$ cells in blood.[33] Furthermore, the long, clinically latent phase that characterizes HIV infection is not a period of viral inactivity, but a steady-state process in which infection, cell death, and cell replacement are kept in balance.[34] The virus replicates at an extraordinarily high rate, and this, inevitably, leads to genetic variation, the emergence of quasispecies, and the accumulation of mutations such as those conferring resistance even before treatment with anti-HIV drugs is started.[35]

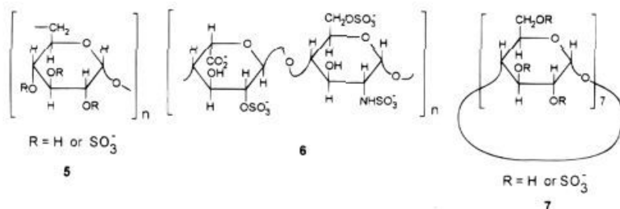
From recent studies with HIV protease inhibitors (i.e. ABT-538 and MK-639), it has become increasingly clear that, *in vivo*, in the HIV-1-infected patient, the composite lifespan of plasma virus and virus-producing cells is remarkably short (half-life $\sim$ 2 days).[36,37] The rapid turnover of plasma virions and CD4 lymphocytes indicate that the replication of HIV-1 is continuous and highly productive, which, in turn, predicts that for treatment strategies to have a dramatic clinical impact, they must be initiated as early in the infection course as possible.[37] Moreover, as the rapid turnover of HIV-1 offers an increased risk for viral escape from therapeutic agents, these agents should be used, either individually or (preferably) combined, at such doses that virus replication is completely suppressed, so as to prevent subsequent rounds of *de novo* virus infection and replication.

As to the different classes of HIV inhibitors which have been most intensively studied, some like the polyanions have been greeted with scepticism, essentially because of lack of evidence of *in vivo* efficacy. Others, like the antisense oligonucleotides, have proved difficult to scale up to therapeutically desirable quantities. Pharmacokinetic problems (poor oral bioavailability, rapid clearance from the bloodstream) have initially hampered the development of the HIV protease inhibitors (although recent progress indicates that these problems may be overcome). The approved drugs AZT, DDC, and DDT suffer from toxic side effects, which may preclude the use of these drugs at doses required to completely suppress virus replication. Emergence of virus-drug resistance, first noted for AZT, has now been observed with virtually any HIV inhibitor that has been used in the clinic or is being considered for clinical use. Particularly, the highly HIV-1-specific non-nucleoside RT inhibitors are notorious for rapidly leading to the development of drug resistance, and this resistance problem has considerably dampened, if not dashed, the hopes for the clinical usefulness of these compounds, at least when used as single agents (i.e. monotherapy).

Virus Adsorption Inhibitors: Polyanionic Substances

Polyanionic compounds, whatever anion [i.e. sulfate as in dextran sulfate (**5**), heparin (**6**), or β-cyclodextrin sulfate (**7**), sulfonate as in suramin (**8**), carboxylate as in aurintricarboxylic acid (**9**), oxometalate as in $H_4SiW_{12}O_{40}$ (JM1493) (**10**)] they are based upon, offer attractive features as anti-HIV agents. They inhibit virus replication in cell culture at a concentration of $0.1-1$ μg/mL, while not being cytotoxic at up to 10 000-fold higher concentration.[38,39] Their mechanism of action can be attributed to interference with virus adsorption, due to a shielding off of the viral envelope glycoprotein gp120 (and, for the polycarboxylates, also a blockade of the cellular CD4 receptor): in particular, the V3 region of gp120 (Figure 1) would serve as the target for the action of the sulfated polysaccharides (i.e. dextran sulfate).[40-42] Consequently, polyanions not only inhibit virus binding to the cells but also virus-induced syncytium formation, which, like virus-cell binding, depends on the interaction of the viral gp120 glycoprotein with the cellular CD4 receptor. Inhibitors of syncytium (i.e. giant cell) formation between virus-infected and uninfected cells may be particularly advantageous in blocking virus transmission through cell-to-cell contact.

Furthermore, the antiviral activity spectrum of the polyanionic substances extends to a variety of enveloped viruses other than HIV, i.e. herpesviruses [herpes simplex virus (HSV), cytomegalovirus (CMV)], ortho- and paramyxoviruses [influenza A, respiratory syncytial virus (RSV)], toga-, flavi-, arena-, bunya- and rhabdoviruses.[43-45] This may make these compounds potentially useful in the treatment of such virus infections, some of which may also be present as opportunistic pathogens in immunosuppressed (i.e. AIDS) patients.



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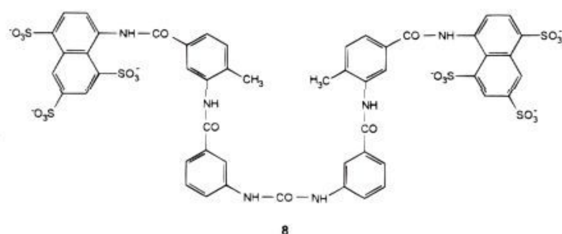

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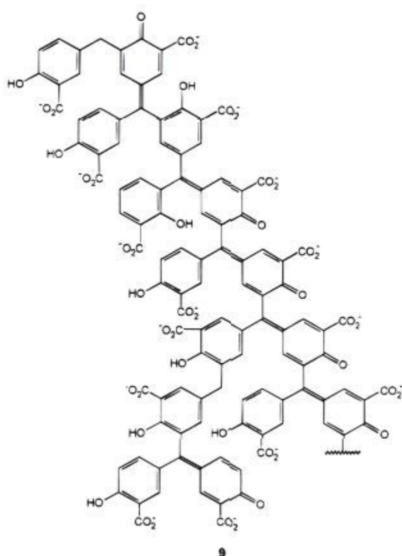

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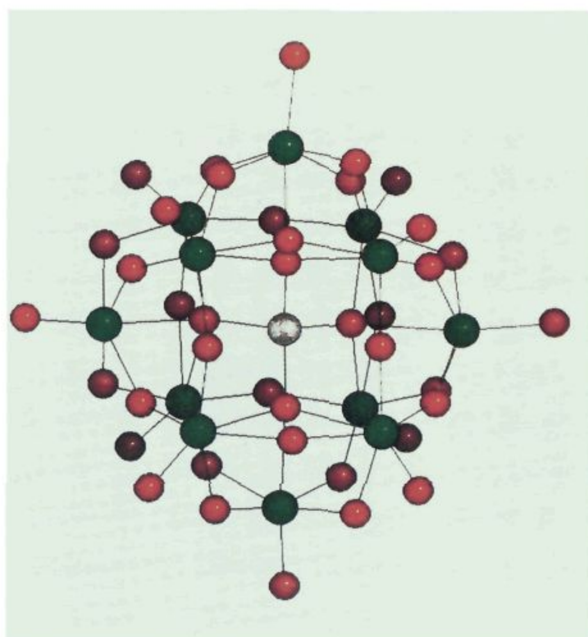

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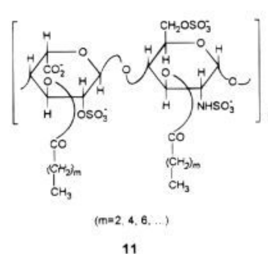
Although it is not known how easily HIV or any of the other enveloped viruses develop resistance to the sulfated polysaccharides or other polyanionic substances, different HIV strains have been shown to differ markedly in their sensitivity to a given compound,[46] and, moreover, different compounds may differ markedly in their antiviral potency and activity spectrum. The latter differences are probably related to differences in the molecular weight and the nature, density, and distribution of the negative charges, whereas the former differences may be attributed to the variability of the molecular target (i.e. V3 loop of the viral gp120 glycoprotein) with which these compounds are assumed to interact.

Polyanionic substances suffer from a number of pharmacokinetic and toxicological drawbacks which seem to compromise their clinical utility. They are poorly absorbed following oral administration,[47] and, even if directly delivered in the bloodstream, they may be retained by various plasma proteins before reaching their site of action. Sulfated polysaccharides are also notorious for their anticoagulant activity, but, as has been demonstrated with periodate-treated heparin[48] and O-acylated heparin (**11**),[49] anti-HIV activity can be achieved with heparin derivatives that have virtually lost their anticoagulant activity. Also, the oral bioavailability of sulfated cyclodextrins can be markedly improved by the appropriate chemical modifications [i.e. substitution of benzyl groups, as in mCDS71 (**12**)].[50,51]



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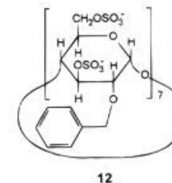

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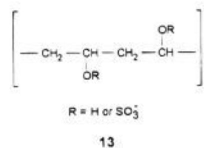

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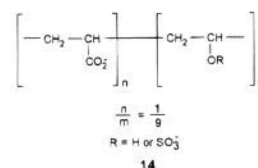

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Many of the problems (i.e. lack of oral bioavailability, anticoagulant or thrombocytopenic activity upon intravenous administration) encountered with the polyanionic substances would seem irrelevant if these compounds were to be given topically, *viz.* in the prophylaxis of sexual transmission of HIV. In fact, topical application of the polyanionic substances [*viz.* polyvinyl alcohol sulfate (PVAS) (**13**) and its copolymer with acrylic acid (PAVAS) (**14**)] have been found to prevent genital HSV-2 infection in mice;[53] follow-up studies with these compounds in the topical prevention of genital FIV (feline immunodeficiency virus) infection in cats are underway.

Virus–Cell Fusion Inhibitors: Lectins, Polypeptides, Negatively-Charged Albumins, and Betulinic Acid Derivatives

Virus–cell fusion can be considered as an attractive target for anti-HIV chemotherapy since the compounds

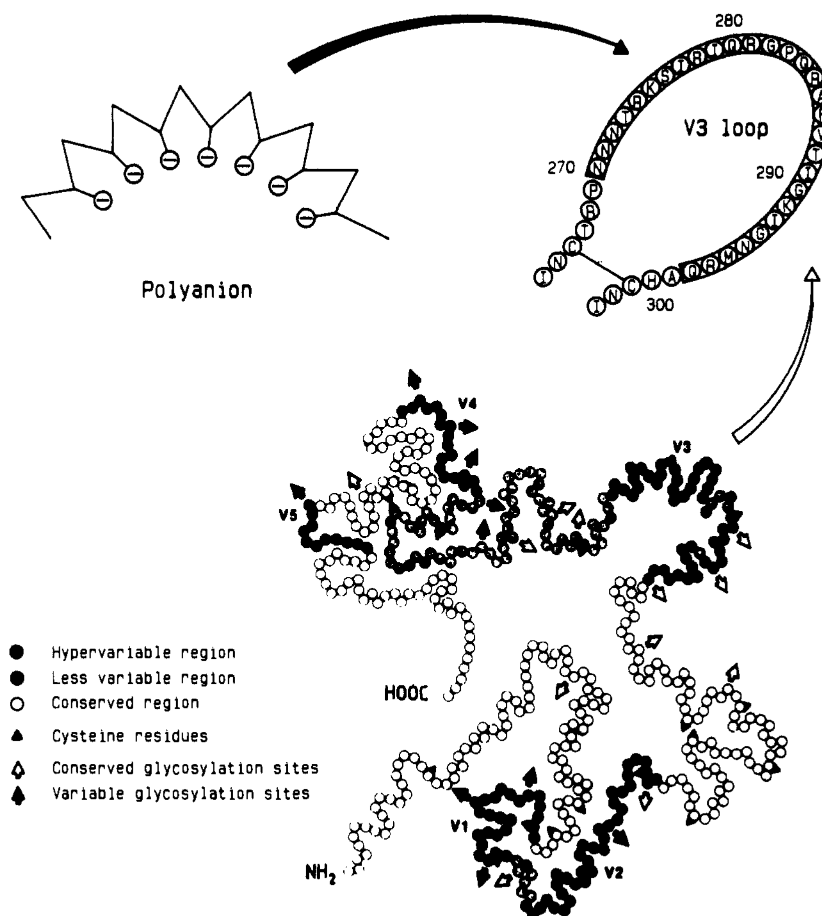
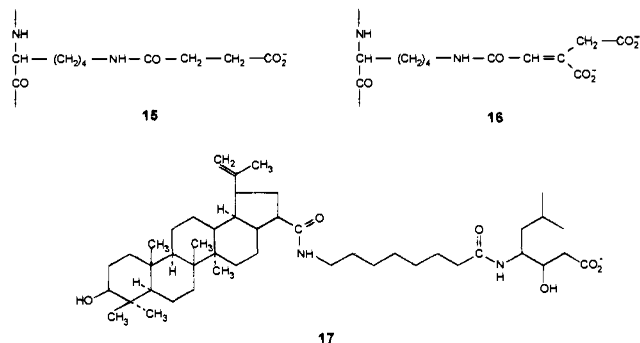


Figure 1. HIV envelope glycoprotein gp120 showing V3 loop, with which polyanionic substances interact.

that interfere with this process may be expected to block viral spread through both virus-to-cell and cell-to-cell contact. A number of compounds have been postulated to interfere specifically with virus–cell fusion: i.e. mannose-specific plant lectins,[53,54] the polypeptide polyphemusin,[55] negatively-charged [succinylated (**15**) or aconitylated (**16**)] albumins[56,57] and triterpene [i.e. betulinic acid (**17**)] derivatives.[58] These compounds (i.e.



aconitylated albumins) inhibit HIV replication at a concentration as low as 0.02 μg/mL, while not being cytotoxic at concentrations up to 1 mg/mL.[57] The compounds were rationalized to interact with the virus–cell fusion process based on the observations that, while inhibitory to syncytium formation between virus-infected and uninfected cells, the compounds did not inhibit virus binding to the cells. Furthermore, their inhibitory effect on syncytium formation closely correlated with their inhibitory effect on virus-induced cytopathicity.

Syncytium formation, as well as virus–cell fusion, depends on the interaction of the envelope glycoproteins gp120 and gp41 with the cell membrane; it is as yet unclear with which region(s) of gp120 or gp41 the fusion inhibitors actually interact. Also, the different fusion inhibitors may interact with different regions of gp120 and gp41. This seems evident from the widely varying chemical structure of the different classes of fusion inhibitors and is further corroborated by the fact that some fusion inhibitors (i.e. plant lectins) are equally inhibitory to HIV-1 and HIV-2, whereas others (i.e. betulinic acid derivatives) are inhibitory to HIV-1 but not HIV-2. Apparently, betulinic acids must interact with an HIV-1 envelope glycoprotein site that is dissimilar from the corresponding epitope in HIV-2.

Synthetic peptides, representing certain domains of the HIV-1 gp41 glycoprotein, i.e. DP-107 and DP-178 (Figure 2),[59] have also proved to be potent inhibitors of HIV-1 infection and virus-mediated cell–cell fusion, which indicates that fusion inhibitors targeted at this transmembrane (TM) glycoprotein have potential as antiviral therapeutics.[60,61] The peptides DP-107 and DP-178 interact with each other, and so do the corresponding domains within the gp41 TM glycoprotein. These molecular interactions should provide the basis for the design of assays aimed at screening potential fusion inhibitors[59] and may well be the site of attack of some of the fusion inhibitors that have been identified so far. It would now seem mandatory to resolve the site(s) of interaction of the different fusion inhibitors: i.e. plant lectins, polyphemusin, negatively charged albumins, and betulinic acid derivatives. In this regard, it

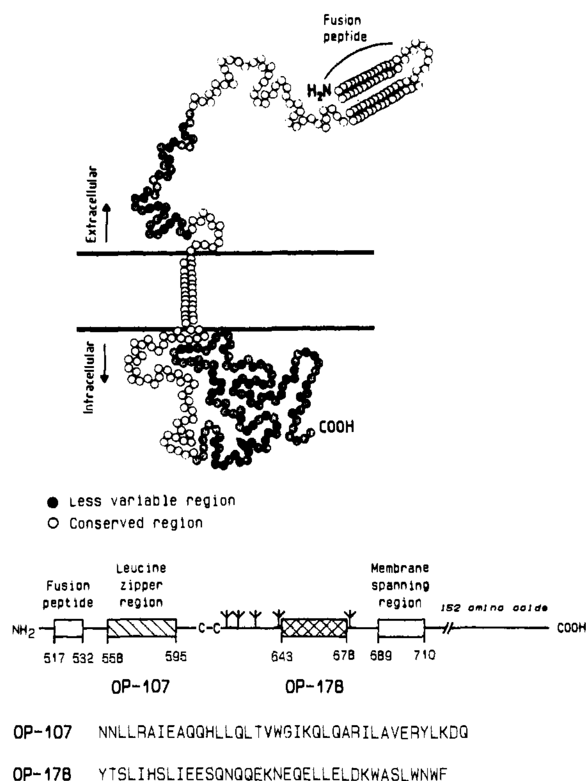


Figure 2. HIV envelope transmembrane glycoprotein gp41. Synthetic peptides DP-107 and DP-178 representing domains of this glycoprotein (according to Petteway *et al.*).[59]

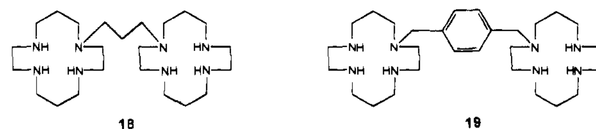
would be of interest to investigate whether the virus readily develops resistance to these fusion inhibitors, as analysis of the amino acid substitutions underlying this resistance may help identify the gp120/gp41 regions with which the compounds interact.

At present, it is difficult to assess the potential clinical usefulness of the virus–cell fusion inhibitors, as the toxicological and pharmacokinetic profiles for most of these compounds remain to be established. The betulinic acid derivatives represent the first low-molecular-weight compounds to be recognized as fusion inhibitors and are prime candidates to be pursued for their potential in the systemic therapy and/or prophylaxis of HIV-1 infections. The negatively-charged albumins may offer some advantages[62] over other polyanionic substances in that they are expected to penetrate well in the lymphatic system, reside for a long time in the bloodstream, possess low immunogenicity and no anticoagulant activity, and be used as carriers for the delivery of other antiviral drugs.

Virus Fusion/Uncoating Inhibitors: Bicyclam Derivatives

Guided by the knowledge of picornavirus (i.e. rhinovirus) uncoating inhibitors, virus uncoating has been regarded as an appropriate target for antiviral agents. Thus, Rossmann[63] speculated that the HIV p24 capsid protein, bearing some resemblance to the VP1 capsid protein of rhinoviruses with which the picornavirus uncoating inhibitors interact, may likewise be a suitable target for HIV uncoating inhibitors. However, no compounds have so far been shown to prevent HIV uncoating through interaction with the p24 antigen. Reviewing the different targets for AIDS therapy,[64] Mitsuya *et al.* suggested that hypericin, an aromatic polycyclic dione (naphthodianthrone) owed its anti-HIV activity to an interaction with the HIV uncoating process. However, the original papers[65,66] did not provide or describe experimental evidence for such uncoating-directed action. In fact, hypericin was shown to directly inactivate HIV and other enveloped viruses, particularly upon illumination by visible light, and thus acts as a virucidal agent.[67,68] When envisaging virus uncoating as a possible target for HIV inhibitors, it should be taken into account that HIV is an enveloped virus and that for enveloped viruses the uncoating process is likely to be more complex than for nonenveloped viruses. If defined as decapsidation, or removal of the viral capsid proteins, uncoating has to be preceeded by the removal of the viral envelope during the fusion process. Yet, these two processes may be functionally and/or structurally linked and should not necessarily be viewed as two independent events.

There is, at present, only one group of compounds that have been postulated to interact with HIV uncoating. These are the bicyclams, represented by JM2763 and JM3100. These molecules consist of two cyclam (1,4,8,-11-tetraazacyclotetradecane) moieties tethered by an aliphatic (i.e. propylene) bridge,[69] as in JM2763 (**18**), or an aromatic [i.e. phenylenebis(methylene)] bridge,[70] as in JM3100 (**19**). The bicyclams JM2763 and JM3100



inhibit HIV-1 and HIV-2 replication within the concentration range of 0.1–1 μg/mL (JM2763) and 1–10 ng/mL (JM3100), while not being toxic to the host cells at concentrations up to 1500 μg/mL.[69,70] In human lymphocytes/macrophages, JM3100 inhibits HIV replication at a concentration of 1 ng/mL (or even lower). It may be considered as one of the most potent and selective HIV inhibitors described to date.

Why have the bicyclams been postulated to be targeted at the viral uncoating process? Time-of-addition experiments, whereby the compounds are added at different times (i.e. 0, 1, 2, 3, ..., up to 24 h) after infection, have indicated that the bicyclams interfere with a stage of the HIV replicative cycle that is intermediate between virus–cell binding and reverse transcription.[69,70] When viral RNA recovered from HIV-infected cells that had been treated with the bicyclam was analyzed for its sensitivity to ribonuclease A, it showed a drug concentration-dependent reduction in degradation, as could be expected if the viral RNA had not been dissociated (i.e. uncoated) from the surrounding viral capsid proteins or envelope glycoproteins.[71]

The bicyclam JM3100 inhibits virus-induced syncytium formation [albeit at a higher concentration (~1 μg/mL) than required for inhibiting viral replication].[70] This points to an action targeted at the fusion process. It is still unknown with which viral glycoproteins and/or capsid proteins, and at which molecular sites, the bicyclams interact. This is the subject of ongoing investigations. One of the targets that the bicyclams may interact with seems to be located in the gp120 V3V4V5-gp41 domain, as suggested by recombination experiments between the wild-type NL-43 clone and a drug-resistant mutant thereof (K. De Vreese *et al.*, in preparation).

NC protein p7

Figure 3. HIV envelope glycoproteins and nucleocapsid (NC) proteins. Primary structure of the NC protein p7, with the two zinc fingers.

One of the capsid proteins that may seem to be an attractive target for antiviral intervention is the p7 protein (Figure 3), which is the last of the capsid proteins to be dissociated from the viral RNA before reverse transcription can take place. The p7 protein contains two zinc fingers[72] which are likely to be involved in the binding of the viral RNA with p7 and which might serve as an interaction point for the bicyclams. Recent studies[73] have indicated that p7 is responsible for packaging the viral RNA by recognizing a packaging site [PSI (ψ)] on the viral RNA genome, and that the N-terminal zinc finger is directly involved in the binding of p7 with PSI. At least one set of compounds, namely the aromatic C-nitroso compounds, have been postulated to achieve their anti-HIV activity through ejection of zinc from the HIV-1 capsid zinc fingers.[74]

Reverse Transcriptase Inhibitors: Substrate Analogues

All the compounds that have so far been approved, or are being considered for approved, for the treatment of HIV infections belong [with the exception of the HIV protease inhibitors, i.e. saquinavir (Invirase)] to the class of the 2′,3′-dideoxynucleoside (ddN) analogues: *viz.* zidovudine (AZT) (**1**), zalcitabine (DDC) (**2**), didanosine (DDI) (**3**), stavudine (D4T) (**4**), and lamivudine (3TC, 2′,3′-dideoxy-3′-thiacytidine) (**20**). At the RT level these ddN analogues act as competitive inhibitors/alternate substrates of the HIV RT. They interact at the substrate-binding site of the enzyme, characterized by its catalytic triad (D110, D185, D186), where the dNTPs normally bind (Figure 4). They act as chain terminators in that following their incorporation into the growing DNA chain they do not permit further chain elongation.[75] To be able to interact with their target enzyme, the ddNs need to be phosphorylated successively to the 5′-monophosphate (ddNMP), 5′-diphosphate (ddNDP), and 5′-triphosphate (ddNTP) form. The initial phosphorylation is a crucial first step in the intracellular metabolism of

Figure 4. Substrate-binding site, characterized by the catalytic triad D110, D185, and D186, at the HIV reverse transcriptase. Also indicated are the locations of the amino acid mutations that confer resistance to the 2′,3′-dideoxynucleoside analogues (positions 41, 67, 69, 70, 74, 89, 184, 215, and 219).

the ddNs.[76] Some ddN analogues [such as DDU (2′,3′-dideoxyuridine)] have a low affinity for the nucleoside kinases [such as thymidine kinase] that are responsible for their initial phosphorylation. Moreover, the nucleoside kinase activity of some (i.e. resting) cells such as monocytes/macrophages may be insufficient to satisfactorily phosphorylate even those ddN analogues (such as AZT) that have high affinity for the enzyme.

20

R=H : PMEA
R=NH_2 : PMEDAP

21

R=H : FPMPA
R=NH_2 : FPMPDAP

22

R=H : PMPA
R=NH_2 : PMPDAP

23

Several strategies could be envisaged to overcome the problem of the initial phosphorylation step. The acyclic nucleoside phosphonates (ANPs) can be considered as ddNMP analogues whereby the first phosphate group has been built in as a phosphonate. These molecules are able to enter the cells, probably by an endocytosis-like process, and, following the attachment of two additional phosphate groups, whereby the ANPs are converted to their corresponding diphosphorylated derivatives, they interact with the RT process. Typical examples of ANPs that act according to this principle are 9-(2-(phosphonylmethoxy)ethyl)adenine (PMEA) (**21**), (*S*)-9-(3-fluoro-2-(phosphonylmethoxy)propyl)adenine (FPMPA) (**22**), (*R*)-9-(2-(phosphonylmethoxy)propyl)adenine (PMPA) (**23**), and their 2,6-diaminopurine counterparts

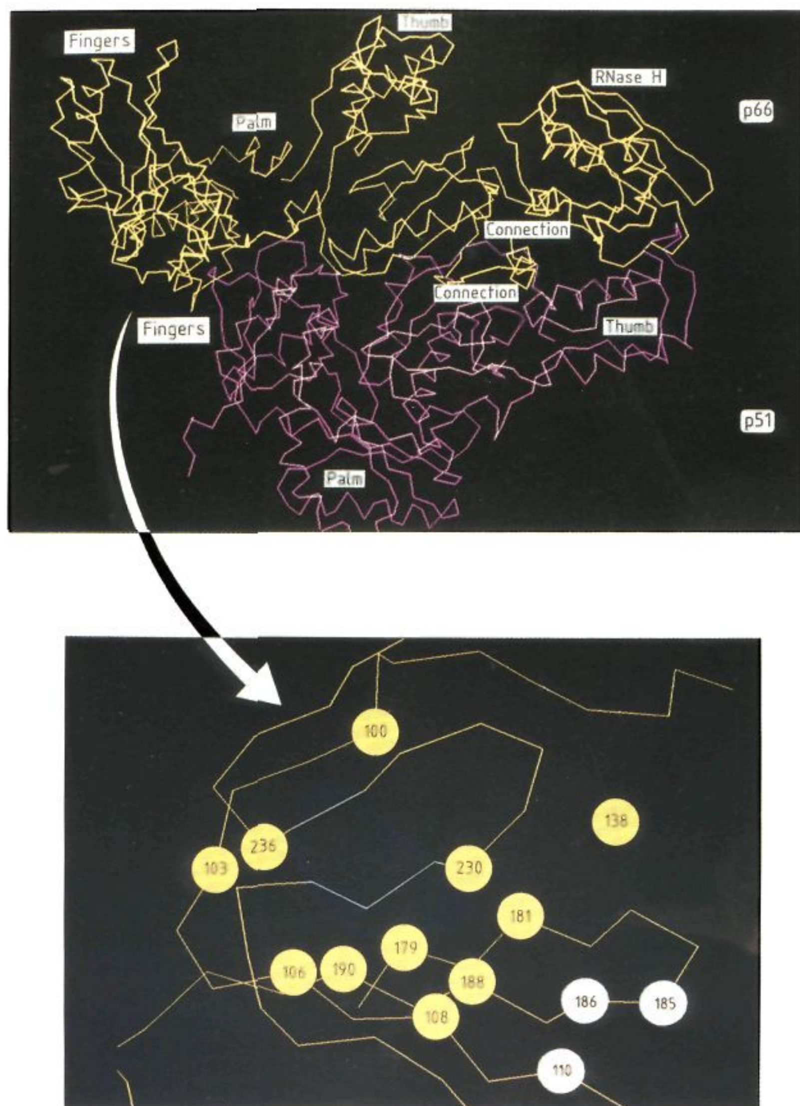


Figure 5. Interaction of NNRTIs with hydrophobic pocket in palm domain of p66 subunit of HIV-1 reverse transcriptase. HIV-1 RT mutations conferring resistance to NNRTIs are located at positions 100, 103, 106, 108, 179, 181, 188, 190, 230, and 236 of the p66 subunit and at position 138 of the p51 subunit. The aspartate residues at position 110, 185, and 186 represent the catalytic triad.

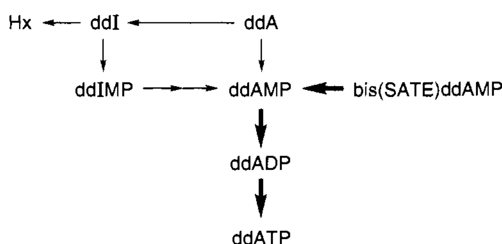
PMEDAP, FPMPDAP, and PMPDAP.[77-79] The intracellular phosphorylation of these compounds can be secured in one step by the PRPP synthetase or in two steps by the AMP kinase. The diphosphorylated derivatives, i.e. PMEApp, FPMPApp, and PMPApp, act at the RT level as competitive inhibitors/substrates with respect to dATP, and if incorporated into the growing DNA chain, they terminate further chain elongation.

PMEA (and some of its congeners) have proved to be effective against a wide range of retroviruses, including murine leukemia/sarcoma viruses, feline leukemia virus, feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) and maedi/visna virus, and hepadnaviruses [hepatitis B virus (HBV)], both *in vitro* and *in vivo*.[80] In fact, PMEA is more efficacious *in vivo* than could have been predicted from its antiviral potency *in vitro*. It is clearly superior to AZT in its *in vivo* efficacy against retrovirus infections (for example, SIV infection in macaques),[81] and this may be related to the long-lasting antiviral action of PMEA and the long half-life of its metabolites (i.e. PMEAp and PMEApp). Additional assets of PMEA (and some of its congeners) are that their activity spectrum may extend to viruses, other than retro- and hepadnaviruses, *viz.* herpes- and papillomaviruses and that they may also exert an immunomodulatory role, i.e. by enhancing natural killer (NK) cell activity.[80]

In attempts to bypass the first, and limiting, phosphorylation step in the metabolic conversion of the ddN analogues to their 5′-triphosphate derivatives (ddNTPs), numerous prodrugs of the 5′-monophosphate (ddNMP) forms have been created, with the hope that these prodrugs would release these ddNMP forms intracellularly. Thus, various cholesteryl, alkyl, dialkyl, aryl, and diaryl phosphate derivatives of AZT and other ddNs (i.e. D4T) were synthesized,[82-84] but most of these prodrugs appeared to act as depot forms of the free nucleoside ddN rather than the nucleotide (ddNMP). However, based on the facts that they are active in TK^- (thymidine kinase-deficient) cells and/or are derived from ddNs that are by themselves inactive (because they are not phosphorylated), the following constructs may be assumed to act as true nucleotide prodrugs: bis((pivaloyloxy)methyl) esters [i.e. bis(POM)ddUMP],[85] bis[*S*-((2-

hydroxyethyl)sulfidyl)-2-thioethyl] esters [i.e. bis(DTE)-ddUMP],[86] aryloxy phosphoramidate derivatives (i.e. *p*-propylphenyl methoxyalaninyl AZTMP)[87] and ddN diphosphate diglycerides.[88] Also, the bis(POM) and bis-(DTE) derivatives of PMEA have been synthesized,[86,89] and as compared to the parent compound PMEA, bis-(POM)PMEA showed increased cellular uptake (*in vitro*) and better oral bioavailability (*in vivo*).[90,91] Other prodrug strategies (i.e. based on the 5′-[4-(pivaloyloxy)-1,3,2-dioxaphosphorinan-2-yl] functionality)[92] that have been worked out for antitumor agents (i.e. FdUMP) may be readily applicable to ddNMP analogues as well.

Intracellular delivery of the ddNMP form may be particularly advantageous for those ddN analogues (i.e. 2′,3′-dideoxyadenosine, ddA) that in their nucleoside form are more rapidly degraded (i.e. by adenosine deaminase) than anabolized by cellular kinases (i.e. adenosine kinase) to their ddNMP form (i.e. ddAMP). Thus, the bis(*S*-acetyl-2-thioethyl)phosphotriester of ddA [bis(SATE)ddAMP] was synthesized and found to be 1000-fold more potent against HIV than the parent compound.[93] This can be readily explained by assuming that direct intracellular delivery of ddAMP leads to a much greater supply of ddATP than if the normal ddA/ddI metabolic pathway would be followed, according to the following scheme:



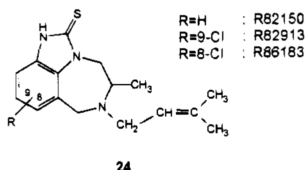
Although all the ddN analogues are assumed to achieve their anti-HIV activity, as competitive inhibitors/alternate substrates for the HIV RT reaction, following their phosphorylation to the corresponding ddNTPs, the intermediary phosphorylated products may also contribute to the biological effects seen with these compounds. In particular, the AZT 5′-monophosphate AZTMP, which is known to accumulate inside the cells,[94] has been accredited with a number of "side" effects: i.e. it inhibits dTTP biosynthesis by interfering with dTMP kinase; it also inhibits the RT-associated RNase H activity[95] and the 3′-exonuclease that would otherwise cleave off AZTMP from the DNA 3′-terminal ends;[96] and, furthermore, AZTMP inhibits protein glycosylation, and this may explain, at least part of, the cytotoxicity of AZT.[97]

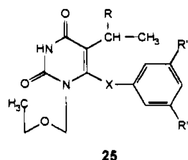
Reverse Transcriptase Inhibitors: Nonsubstrate Analogues

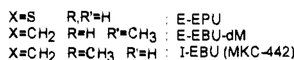
While the ddN analogues, following their conversion to the corresponding ddNTPs, act as competitive inhibitors/substrates with regard to the natural substrates (dNTPs), a number of compounds, commonly referred to as nonnucleoside reverse transcriptase inhibitors (NNRTIs),[98,99] interact non-competitively with an allosteric site, thereby inactivating the enzyme. In fact, the NNRTIs (i.e. BHAP, U-90152E) show a significantly higher binding affinity for the enzyme–substrate complex than for the free enzyme and consequently do not directly impair the function of the substrate binding site.[100] NNRTIs are only active against HIV-1 RT. They are inactive against HIV-2 RT or any other retrovirus-associated RTs. This unique specificity of the NNRTIs for the HIV-1 RT is due to the presence in HIV-1 RT, but not other RTs or DNA polymerases, of a flexible, highly hydrophobic pocket in which the NNRTIs must fit snugly.[101–103] The study of drug-resistant HIV-1 RT has resulted in the identification of the crucial amino acids that take part of this pocket and determine the binding of the NNRTIs with their target site, *viz.* the amino acid residues 100 Leu, 103 Lys, 106 Val, 108 Val, 138 Glu, 179 Val, 181 Tyr, 188 Tyr, 190 Gly, 230 Met, and 236 Pro (Figure 5). Mutations at these positions, i.e. 100 Leu → Ile, 103 Lys → Asn, 106 Val → Ala, 108 Val → Ile, 138 Glu → Lys, 179 Val → Asp, 181 Tyr → Cys, 188 Tyr → Cys/His, 190 Gly → Glu, 230 Met → Ile, and 236 Pro → Leu, lead to resistance of the HIV-1 RT to one or more NNRTIs.[104,105] The role of these amino acids may be either direct in that they could be directly involved in the binding of the NNRTIs to the HIV-1 RT or indirect in that they may contribute to the conformation that is optimal for NNRTI binding. All mutations that have been shown to confer resistance to NNRTIs cluster around the pocket where the NNRTIs bind, suggesting that these resistance mutations lead to a direct alteration of inhibitor binding.[106] Of all the amino acids that have been found to lead to resistance to NNRTIs when mutated, only one (138 Glu → Lys) is located on the p51 subunit;[107,108] all the others are part of the p66 subunit.

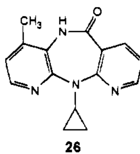
The binding of the NNRTIs to their hydrophobic pocket of the HIV-1 RT does not interfere with the binding of the dNTPs but slows down the rate of incorporation of the dNTPs (as dNMPs) in the DNA product.[109] Because of the cooperative interaction between the substrate-binding site nonsubstrate NNRTI-binding site, combination of the functionalities of a non-nucleoside and nucleoside type of RT inhibitor was postulated to result in a very tight binding to the HIV-1 RT.[109]

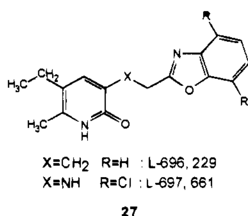
The tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives (**24**) and 1-[(2-hydroxyethoxy)methyl)]-6-(phenylthio)thymine (HEPT) derivatives (**25**) were the first compounds to be reported as specific HIV-1 inhibitors targeted at the viral RT.[110–113] They were discovered through the evaluation in cell culture of the anti-HIV activity of a variety of different chemical entities. Soon after the discovery of TIBO and HEPT, other compounds, i.e. nevirapine (**26**), pyridinone (**27**), and bis(heteroaryl)piperazine (BHAP) (**28**), emerged as HIV-1-specific inhibitors from screening programs for inhibition of HIV-1 RT activity. Subsequently, the list of NNRTIs was extended to various other classes of compounds,[98,99] including α-anilinophenylacetamide (α-APA) derivatives [i.e. R89439 (loviride) (**29**)], 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-3′-spiro-5″-(4″-amino-1″,2″-oxathiole)-2″,2″-dioxide (TSAO) derivatives (**30**), (phenylethyl)thioureathiazole (PETT) derivatives [i.e. LY297345 (**31**)], quinoxaline S-2720 (**32**), dihydrothiazoloisoindolones (**33**), imidazodipyridodiazepines (**34**), 5-chloro-3-(phenylsulfonyl)indole-2-carboxamide [L-737,-126 (**35**)], pyrrolobenzothiazepines,[114] 3,4-dihydro-2-alkoxy-6-benzyl-4-oxopyrimidines (DABOs),[115] highly substituted pyrroles,[116] and yet others.[117] While all these compounds may be assumed to bind to the same

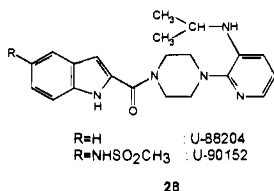


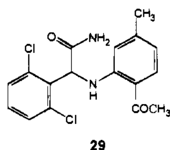


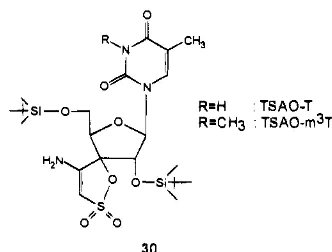


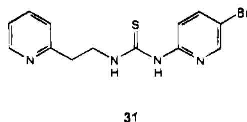


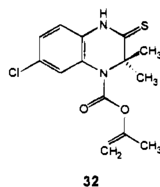


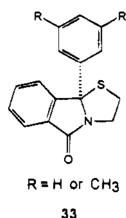


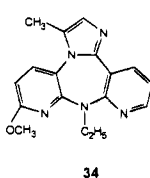


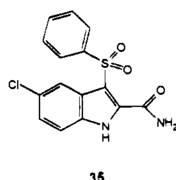












pocket site at the HIV-1 RT, different classes of HIV-1-specific RT inhibitors may differ from one another with regard to the exact amino acid residues of the pocket site with which they interact. The latter conclusion can be drawn from the fact that different NNRTIs do not necessarily show cross-resistance to each other. The mutations that confer resistance to the different NNRTIs may overlap, only partially overlap, or not overlap at all.[104] Furthermore, structural modifications of existing NNRTIs may afford derivatives with a sensitivity/resistance profile that is different from that of the parent compounds, as exemplified by moving the chlorine from the 9 position of the benzodiazepine ring (R82913) to the 8 position (R86183) (**24**); this relatively minor chemical modification restores activity against the 181 Tyr → Cys mutant which is resistant to the 9-chloro derivative.[118]

NNRTIs demonstrate a number of properties which make them potentially more useful than the ddN analogues as anti-HIV drugs: (i) they do not require previous phosphorylation which could otherwise compromise their anti-HIV activity in metabolically resting cells; (ii) they act noncompetitively with respect to the natural substrates at the RT and, therefore, their action cannot be endangered in metabolically active cells by high pool levels of the natural substrates (dNTPs); (iii) they achieve a unique specificity, as they interact with a molecular site at the HIV-1 RT that does not occur at other DNA polymerases, while, in contrast with the NNRTIs, the ddNTPs should be able to interact with all kinds of DNA polymerases, as they are targeted at the substrate-binding site; (iv) due to this unique specificity for the HIV-1 RT, NNRTIs achieve an inhibition of HIV-1 replication in cell culture at nM concentrations, and, while not being toxic to the host cells at concentrations up to 0.1−1 mM, they reach selectivity indexes [100 000-fold (or higher)] that exceed those generally obtained by the ddN analogues; (v) admittedly, NNRTIs seem to lead more rapidly to the emergence of virus−drug resistance than do the ddN analogues, but this problem can be prevented, at least *in vitro* (cell culture), if from the beginning the NNRTIs are used at sufficiently high (but still nontoxic) concentrations; under these conditions, the NNRTIs completely suppress virus replication and prevent the breakthrough of any virus, whether resistant or not.

As has been demonstrated with several NNRTI representatives[119] (i.e. TIBO, HEPT, nevirapine, pyridinone, and BHAP), these compounds are able to completely suppress virus replication in cell culture for at least three months (and probably longer),[120] while under the same conditions, ddN analogues (i.e. AZT) cannot prevent the virus from breaking through even after a few days in the continued presence of the compound.[120,121] To achieve this apparent clearance of virus from virus-infected cells, the NNRTIs have to be added from the start at a sufficiently high concentration (≥100-fold their 50% antivirally effective concentration), which for the individual compounds may vary from 0.1 to 10 μg/mL. Using this "knocking-out" procedure, the cells can apparently be cleared from the virus, as evidenced by several parameters (i.e. virus-induced cytopathicity, viral p24 antigen production, and, most importantly, the presence of proviral DNA).[119] This "knocking-out" phenomenon has been observed *in vitro*, with selected HIV-1 strains (i.e. III_B) in selected cell culture systems (i.e. CEM cells). This phenomenon needs to be confirmed for various other cell types, including peripheral blood lymphocytes and monocytes/macrophages, and, of course, it remains to be established whether these compounds can completely suppress virus replication *in vivo*, in the clinic. It should be pointed out that the concentrations required for the NNRTIs to knock out HIV-1 *in vitro* should be therapeutically feasible *in vivo* without risk for toxic side effects.

HIV Integrase as a Target for HIV Inhibitors

The HIV integrase is an attractive target for selective anti-HIV therapy since there is no known functional counterpart in human cells. The only enzyme required for HIV-1 integration is the integrase (IN), a protein of 32 kDa encoded at the 3′ end of the *pol* gene (for a review, see Katz and Skalka, 1994).[122] The enzyme is produced by protease-mediated cleavage of the *gag-pol* precursor during virion maturation. Integrase recog-

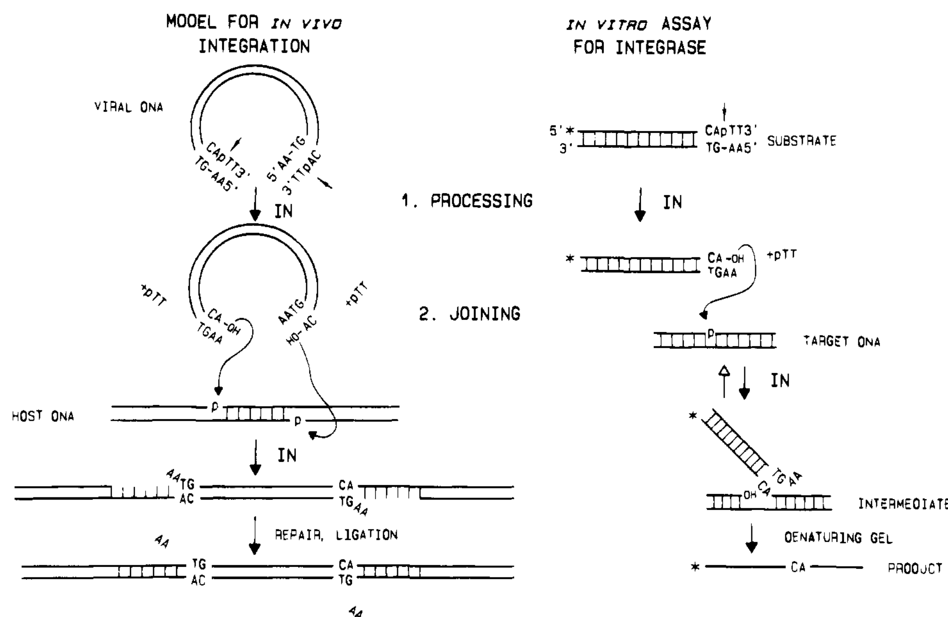


Figure 6. *In vivo* model and *in vitro* assay for retroviral DNA integration, as exemplified for MLV (according to Katz and Skalka).[122] The asterisks indicate a radioactive 5'-phosphate. IN, integrase.

nizes specific sequences in the LTRs of the viral DNA copy: 5'-AC**TG**...**CA**GT-3'. The **TG**...**CA** repeat has been conserved throughout evolution. In the first step of the integration reaction (Figure 6), termed 3' processing, two nucleotides (i.e. pTT) are removed from each 3' end to produce new 3' hydroxyl ends (**CAOH**-3'). This reaction occurs in the cytoplasm, presumably in association with a subviral structure. After entering the nucleus, the processed viral DNA is joined to host target DNA. The joining reaction includes a coupled 4−6 bp staggered cleavage of the target host DNA and the ligation of processed **CAOH**-3' viral DNA ends to the 5' phosphate ends of the target DNA. Repair of the remaining gaps is probably accomplished by host enzymes. Oligonucleotide-based assays[123] have been designed to mimic both processing and joining reactions *in vitro* (Figure 6). HIV IN is composed of three functional domains.[122] The N-terminal region is characterized by a HHCC "zinc finger"-like sequence. The central region is characterized by three highly conserved amino acid residues D,D (35)E and encompasses the catalytic domain for both processing and joining activities. The C-terminal domain seems to be important for binding to the HIV LTR DNA region.

A number of approaches could be considered in attempts to interfere with HIV-1 integration: (i) triple helix-mediated inhibition, (ii) inhibition by peptides from combinatorial peptide libraries, and (iii) screening of chemical libraries and natural compounds. The integrase-binding site located in the LTR region contains a purine motif, 5'-GGAAGGG-3', that can be selectively targeted by oligonucleotide−intercalator conjugates.[124] Under neutral pH and at physiological temperature, these conjugates readily form a stable complex with the viral DNA, thus giving rise to a short DNA triplex. It has been shown that this triple-helix formation can prevent the catalytic functions of the integrase *in vitro*. However, this elegant approach is complicated by the difficulties encountered in intracellularly delivering the conjugates and furthermore jeopardized by the high mutation rate of HIV that may lead to base substitutions in the LTRs. Screening of synthetic peptide combinatorial libraries in the oligonucleotide-based microtiter plate assay has recently allowed the identification of an hexapeptide (i.e. HCKFWW) as an inhibitor (IC_{50} 2 μM) of integrase activity.[125]

Several DNA binding agents were found to inhibit HIV-1 integrase, probably due to a nonspecific interaction with the DNA binding domain of the enzyme.[126] Polyhydroxylated aromatic compounds (i.e. catechol derivatives) have been postulated to interact with the catalytic domain of the HIV-1 integrase, possibly by interfering with the coordination of the metal ions that are required for the phosphoryl transfer reactions.[127] However, the catechol derivatives do not exhibit much antiviral specificity in cell culture and are no longer considered to be worth pursuing.[128]

The recently established high-throughput microtiter plate assays,[129,130] on the one hand, and the elucidation of the three-dimensional structure of the catalytic domain of HIV-1 integrase, on the other hand,[131] will boost the antiviral screening of chemical libraries as well as the structure-based design of integrase inhibitors. Finally, the human gene product (termed INI1) was recently found to cooperate with HIV-1 integrase[132] in targeting integration of the viral DNA into actively transcribed genes of the human cell genome. This may open new perspectives for the development of drugs that interfere with this protein−protein interaction.

Different Sites of Interaction: Antisense Oligonucleotides

Antisense oligonucleotides are generally looked upon as inhibitors of viral mRNA translation because of their capacity to form stable duplexes with complementary sequences of the viral mRNA. A representative example is GEM 91, a 25-mer complementary to the HIV-1 *gag* mRNA initiation site (Figure 7). GEM 91 may thus block HIV replication through hybridization, followed by translation arrest from the *gag* mRNA initiator codon.[133] Phosphodiester-, phosphorothioate-, and phosphorodithioate-based oligo(deoxy)nucleotides, once they have been hybridized to their target mRNA, may rely

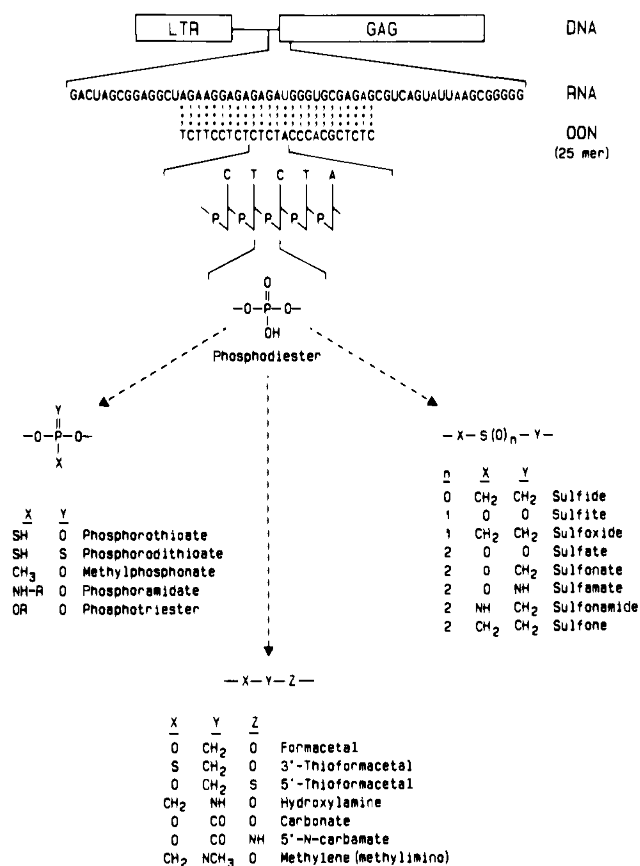


Figure 7. Antisense oligonucleotides. For example:[133] GEM 91, a 25-mer oligodeoxynucleotide phosphorothioate, complementary to the *gag* mRNA of HIV-1 at the initiator codon (AUG). In attempts to increase cellular permeation of antisense oligonucleotides, protect them against degradation by cellular nucleases and/or enhance their affinity for their target nucleotide sequences, the natural phosphodiester linkage can be replaced by various other linkages (i.e. phosphorothioate, phosphorodithioate, etc.).[134]

on the cellular ribonuclease H to cleave the RNA, which allows a single oligo(deoxy)nucleotide to eliminate multiple copies of the target mRNA. However, not all oligonucleotides are competent for RNAse H-activated cleavage of RNA: only the phosphodiester-, phosphorothioate-, and phosphorodithioate-based oligonucleotides are, whereas the methylphosphonate-, phosphoroamidate-, and many other backbone-modified oligonucleotides are not.[134] If the latter oligonucleotides are effective in inhibiting mRNA translation, they must do so by sterically blocking the mRNA, i.e. preventing its interaction with the cellular components required for mRNA translation.

Antisense oligonucleotides may be targeted at specific elements of the viral RNA, i.e. TAR and RRE [that are recognized by the regulatory proteins Tat and Rev (see below)], and thus disturb the virus regulatory machinery. Antisense oligodeoxynucleotides can also be designed to form DNA triple helices with specific proviral DNA target sequences, and such oligonucleotides may be expected to inhibit transcription of the viral mRNA in cells carrying the HIV proviral DNA genome.[135] In principle, antisense oligo(deoxy)nucleotides could be targeted at any region of the proviral DNA genome. Such antisense oligo(deoxy)nucleotides could be equipped with a specific DNA-cleaving functionality, and provided they readily reach their target within the cell and engage in the formation of a stable triplex, they may be envisaged to be able to remove the targeted genes from the genome. Antisense oligo(deoxy)nucleotides may also be targeted at the integration process. Therefore, the oligonucleotides should engage in triple-helix formation with the duplex proviral DNA sequences that are recognized by the viral integrase. Antisense oligonucleotides can also be designed to block the reverse transcription process, i.e. through binding to the primer-binding site of the viral RNA (as has been achieved particularly with a phosphorodithioate-based 14-mer complementary to this site.[136] Antisense oligo(deoxy)nucleotides could also block the reverse transcription process by a ribonuclease H-dependent mechanism;[137] irrespective of the site where the oligonucleotide became attached to the viral RNA, it may allow the RNA sequences where it is bound to be cleaved by the RT-associated ribonuclease H. Finally, antisense oligonucleotides that are based on a negatively charged backbone, because of their polyanionic character, may also be expected to inhibit virus adsorption, just as do all other polyanionic substances (polysulfates, polysulfonates, polycarboxylates, and polyoxometalates).

However, the main purpose of the antisense oligonucleotides is not to inhibit virus adsorption, which can be accomplished by less sophisticated polyanions but to block HIV replication at the transcription/translation level. The antisense oligonucleotides should meet a number of requirements:[138] they should be easily synthesized in bulk at reasonable cost, they should be readily bioavailable, preferably by the oral route, they should be able to enter (and to be retained by) the target cell, they should be resistant to degradation by nucleases, and they should not interact in a non-sequence-specific manner with any macromolecules, but, on the other hand, should have high affinity for their ultimate target sequence at the RNA or DNA level. Although modification of the phosphodiester backbone has been shown to impart stability and may also allow for enhanced affinity and increased cellular permeation,[134] the "ideal" antisense oligonucleotide that optimally meets all three requirements still needs to be constructed.

Ribozymes: A Paradigm for Gene Therapy?

Ribozymes could be considered as a special class of antisense oligo(ribo)nucleotides that, following hybridization with their target RNA, cleave a specific phosphodiester bond in this target RNA (Figure 8). Most of the ribozymes that have been constructed are of the "hammerhead" type,[139] but some others follow the "hairpin" type.[140] A ribozyme can be targeted at any site of the viral RNA, where it will engage in the formation of two double helical RNA stretches before cleaving, and thus destroying, the target RNA. As for antisense oligonucleotides in general, the effectiveness of ribozymes will depend on their cellular uptake, stability to nucleases, and affinity/specificity for their target RNA. Ribozymes can be delivered exogenously to the cells,[141] and this delivery can be enhanced by electroporation, conjugation to polycations, and encapsulation in liposomes.[142] Their stability toward nucleases can be increased by the appropriate chemical modifications that leave the catalytic efficiency virtually intact.[143] Tethering the ribozyme to the HIV packaging signal may enhance the ribozyme's efficiency by colocalizing it with the HIV mRNA transcripts inside the

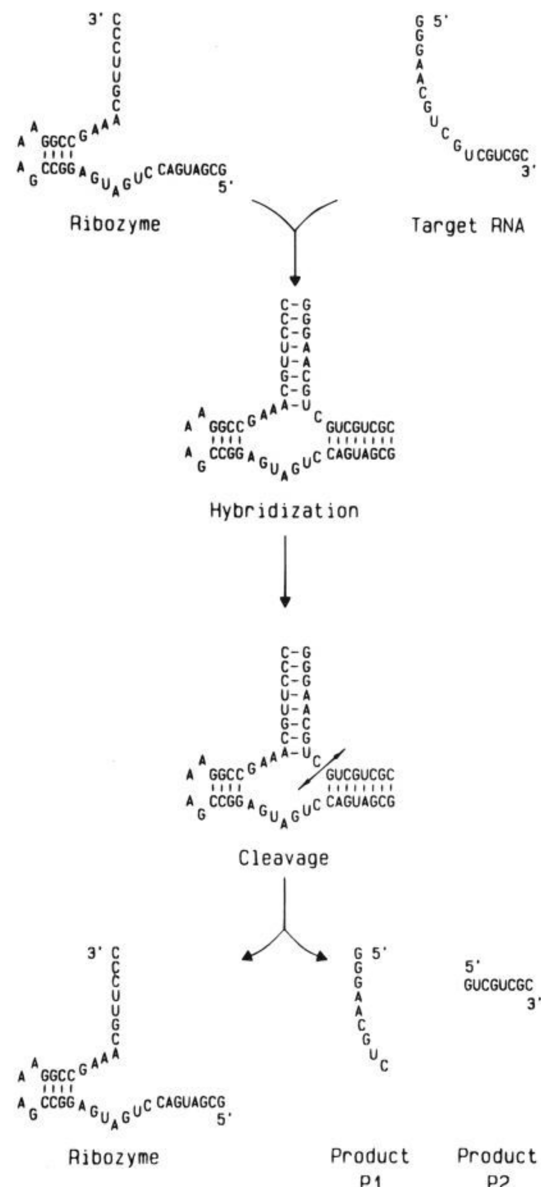


Figure 8. Hammerhead ribozyme hybridizes to a specific RNA sequence, containing GUCG, and cleaves it between C and G in two products.

cell.[144] Yet, the "ideal" ribozyme that unites all desirable features of delivery, specificity, stability, efficiency and accessibility still has to be constructed.

As to delivery, antisense oligonucleotides in general, and ribozymes in particular, may be introduced in the cells *via* (retro)viral vectors.[145,146] This would then allow constitutive expression of the antisense oligonucleotide or ribozyme, leading to inhibition of HIV gene expression in the cell that has already been infected by HIV as well as conferring "intracellular immunity" of noninfected cells against subsequent HIV infection.[140] "Intracellular immunization" of hematopoietic stem/progenitor cells with an anti-HIV-1 ribozyme has proved feasible;[147] thus, ribozyme gene therapy using stem cells as targets could be considered as a promising preemptive strategy for the treatment of HIV infection.

The efficiency and specificity of ribozymes in cleaving their target RNA may be enhanced by the p7 nucleocapsid protein,[148] and gene therapy approaches could be elaborated whereby ribozymes and such proteins are

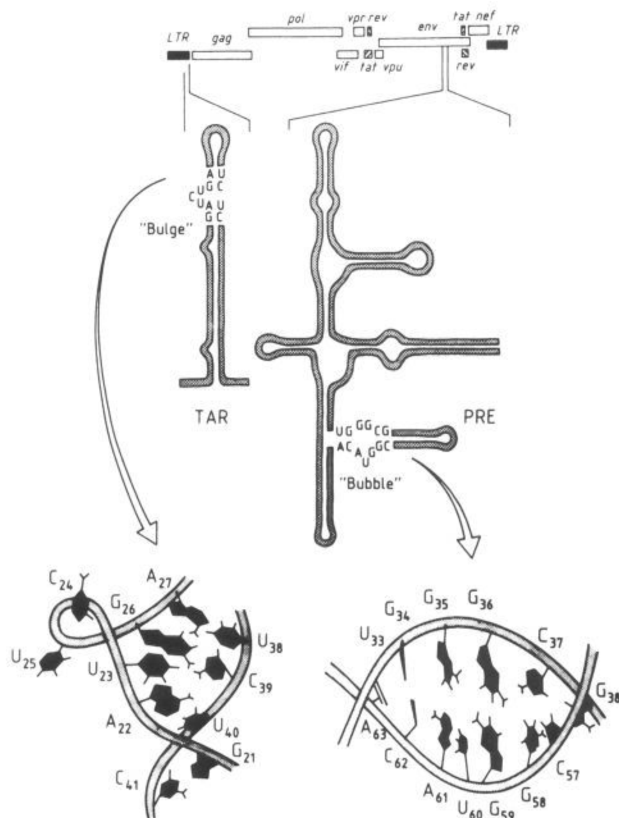


Figure 9. RNA elements TAR and RRE that are recognized by the HIV regulatory proteins Tat and Rev, respectively. Tat-binding site on TAR RNA contains a "bulge", whereas Rev-binding site on REE RNA contains a "bubble" (according to Gait and Karn[149]).

jointly expressed and thus jointly confer intracellular immunization. Also, constructs could be envisaged whereby the ribozyme is covalently linked to antisense oligonucleotides or to the 3′-end of the tRNA primer. Such constructs may be targeted at the 5′-end of the HIV RNA (where the tRNA primer normally binds) and cleave the viral RNA as soon as it has hybridized with the primer binding site. Also in this case the ribozyme efficiency in cleaving the viral RNA could be further enhanced by the presence of the p7 nucleocapsid protein.[146,148]

Regulatory Proteins Tat and Rev as Therapeutic Targets

The HIV regulatory proteins Tat and Rev play an important role in the expression of the HIV genes: Tat by stimulating transcription from the viral long terminal repeat (LTR), and Rev by increasing the transport/stability of the late mRNAs encoding the structural proteins of the virus. Both proteins achieve their effect by a highly specific binding to *cis*-acting regulatory elements in the viral mRNAs. Tat activity requires binding to the *trans*-activation-responsive region (TAR), a stem−loop structure found at the 5′-end of the viral mRNA (located downstream of the transcriptional initiation site in the LTR region), whereas Rev activity requires binding to the Rev-responsive element (RRE), a region rich in stem−loops located within the coding sequence of the *env* gene (Figure 9). Within the TAR and RRE elements, specific regions have been implicated in the direct recognition by the Tat and Rev

proteins:[149] a "bulge"-like structure within TAR, and a "bubble"-like structure within RRE. The identification of the TAR bulge and RRE bubble as the recognition sites for Tat and Rev raises the exciting possibility that inhibitors might be found that interfere with the binding of Tat and Rev to the TAR bulge and RRE bubble, respectively. There may also be some prospects for specific drug design, since the three-dimensional structures of the binding sites on TAR and RRE RNA should soon be known.[149]

In marked contrast with the elegant insight in the mode of interaction of the Tat and Rev proteins with their specific RNA recognition points is the paucity of compounds that have been found to inhibit Tat or Rev. The best known Tat inhibitors are Ro 5-3335 [7-chloro-5-(2-pyrryl)-3*H*-1,4-benzodiazepin-2(*H*)-one] (**36**)[150] and its congener Ro 24-7429 (**37**), in which the NH-CO functionality has been replaced by the N=C($NHCH_3$) functionality.[151] In addition, some keto/enol epoxy steroids have also been reported to act as HIV-1 Tat inhibitors.[152] The Tat inhibitors Ro 5-3335 and Ro 24-7429 are inhibitory at a concentration of about 1 μM to both HIV-1 and HIV-2 infection. They are active against both acute and chronic HIV infection, which is to be expected from compounds that would interfere with a postproviral DNA integration stage. However, in some cell systems (i.e. MT-4 cells, cytokine-stimulated T cells) HIV may replicate independently from tat transactivation (but dependently on the NF-kB transactivation), and under such conditions, tat antagonists (such as Ro 24-7429) fail to suppress HIV-1 replication.[153] This may explain at least in part the discouraging results of the preliminary clinical trial with this same tat antagonist Ro 24-7429.

While active against AZT-resistant HIV strains (and probably NNRTI-resistant strains as well), the Tat inhibitors Ro 5-3335 and Ro 24-7429 themselves do not lead to the development of resistance, even after prolonged (2-year) exposure in cell culture.[151] This is not surprising, since this class of compounds may be assumed to interact with one of the cellular factors involved in the transactivation process rather than the Tat protein *per se*.[154] Marked differences have been observed in the anti-HIV activity and cytotoxicity of Ro 5-3335, depending on the cell type used, and this points to a cellular, rather than a viral, protein as target site for Ro 5-3335. Various cellular factors are known to cooperate with Tat in the overall transactivation process;[155] some of these factors may bind directly to the proviral DNA near the transcription initiation site, whereas others, like a serine/threonine kinase, may interact directly with the Tat protein.[156] It is doubtful that sufficient selectivity for the HIV replicative cycle could be achieved by inhibitors targeted at any of the cellular factors involved in the transactivation process.

The situation is not much different for inhibitors of the Rev protein. Inhibition of the interaction of Rev with RRE has been reported for pyronin Y,[157] an intercalating cationic dye with specificity toward RNA rather than DNA. Yet, despite its strong inhibitory effect on Rev−RRE complex formation, pyronin Y did not inhibit HIV-1 replication and proved too toxic to the host cells.[157] Recently, a series of 8-alkyl-2-(4-pyridyl)-pyrido[2,3-*d*]pyrimidin-5(8*H*)-ones have been described as inhibitors of the cellular Rev response.[158] These

Figure 10. HIV-1 protease-mediated cleavage of the viral *gag* and *gag-pol* precursor proteins. HIV-1 protease inhibitors conceived as peptidomimetics of the transition state formed during hydrolysis of the peptide linkage.

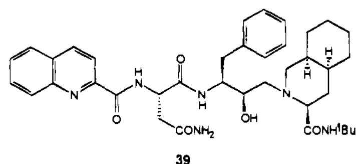
compounds proved inhibitory to HIV-1 replication at roughly the same concentration as that required to inhibit the Rev response. WIN 49569 (**38**), the most

potent compound of this series, inhibited Rev and HIV at an IC_{50} of 2−3 μM, while cytotoxicity was observed at a 10-fold higher concentration (25 μM).[158] These are the first reported small-molecule inhibitors of Rev which also inhibit HIV-1 replication. They may be considered as leads for the design of new derivatives that are more potent and/or less cytotoxic inhibitors of HIV replication.

Protease Inhibitors

The elucidation of the three-dimensional structure[159] of the HIV protease dimer, the advanced knowledge of the class (i.e. aspartyl) of proteases to which the HIV protease belongs, and the development of rapid enzyme assays for screening HIV protease inhibitors have greatly facilitated the design and development of such inhibitors. The HIV protease cleavage sites in the *gag* and *gag-pol* precursor proteins (Pr 55 and Pr 160, respectively) have been identified (Figure 10). In designing HIV protease inhibitors, the "transition-state peptidomimetic" principle was followed, which means that in the inhibitors the hydrolyzable peptide linkage

was replaced by a nonhydrolyzable transition-state isostere, i.e. hydroxyethylamine [as in Ro 31-8959 (**39**)],[160] hydroxyethylene [as in U-81749 (**40**) and L-687,-908 (**41**)],[161,162] (*R*)-(hydroxyethyl)urea [as in SC-52151 (**42**)],[163] norstatine [as in KNI-227 (**43**)],[164] and the C_2 symmetric monoalcohol [i.e. A-74704 (**44**)][159,165] and diol (dihydroxyethylene) [i.e. A-77003 (**45**)][166] and amino diol



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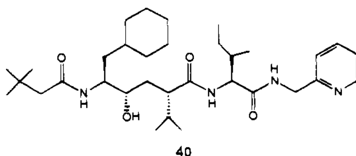

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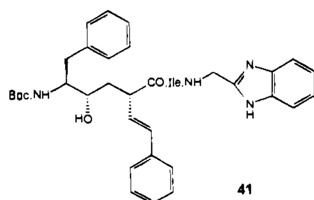

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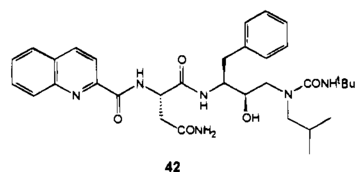

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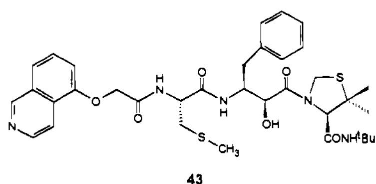

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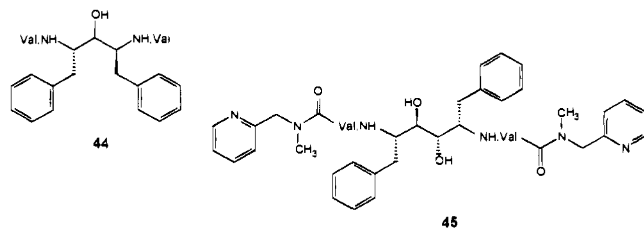

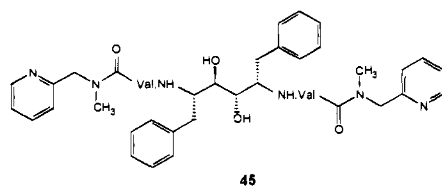

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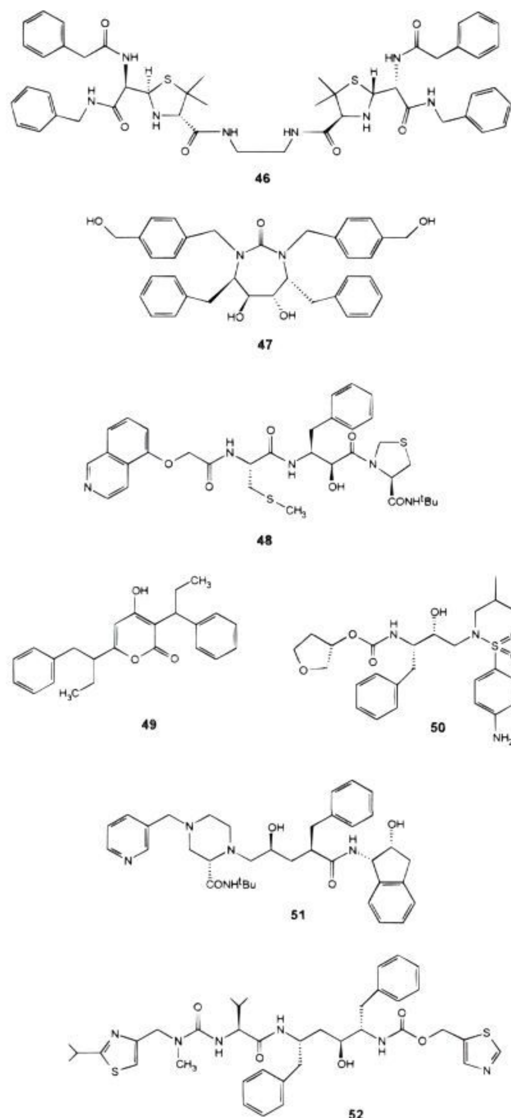
derivatives.[167] Various HIV protease inhibitors containing the dihydroxyethylene transition-state isostere have been synthesized, and, starting from Ro 31-8959 as the model compound, various new ligands were introduced at either the P_2 or P_3 position of the molecule. Constrained "reduced amide" type inhibitors of HIV protease have been constructed whereby three amino acid residues of the polypeptide chain were locked into a g-turn conformation.[168]

Several HIV protease inhibitors [i.e. Ro 31-8959 (**39**) and A-77003 (**45**)] have been the subject of extensive preclinical evaluation. These compounds offer interesting perspectives as candidate anti-HIV drugs, i.e. Ro 31-8959 is active against HIV-1 in cell culture at a concentration of 1−2 nM and inhibitory to the HIV-1 protease at a K_i of 0.1 nM. It is not inhibitory to renin, pepsin, cathepsin, elastase, prolidase, or collagenase. It is active in both acutely and chronically HIV-infected cells[169] and, as could be anticipated, also active against HIV strains that are resistant to AZT or other RT inhibitors. However, most HIV protease inhibitors, and peptide-based drugs in general, have poor oral bioavailability and a short half-life in the bloodstream. The HIV protease inhibitor Ro 31-8959 would be an exception to this rule, as it achieves plasma levels upon oral administration that for several hours exceed those concentrations that are required to inhibit HIV replication *in vitro*. For those HIV protease inhibitors that are too hydrophobic and too rapidly cleared from the bloodstream, phosphate groups could be introduced *via* the hydroxyl group of the serine or threonine residues so as to make them more water soluble and to maintain higher blood levels *in vivo*.[170] Better oral bioavailability can be achieved by low-molecular-weight peptidyl aldehyde inhibitors of HIV protease.[171]

As an alternative to the peptide-based approach, penicillin-derived compounds have been pursued as HIV protease inhibitors, i.e. penicillin C_2 symmetric dimers held together by an ethylenediamine linker (**46**),[172] and monomeric penicillins linked to peptide isosteres such as statine.[173] On the basis of the knowledge of the X-ray crystal structure of the HIV protease dimer, an entirely new class of HIV protease inhibitors, that of the nonpeptide cyclic ureas, has been designed.[174,175] The prototype of this series of HIV protease inhibitors, DPM323 (**47**), inhibits the enzyme at a K_i of 0.27 nM and HIV-1 replication *in vitro* at an IC_{50} of 0.036 μM, and in contrast with most of the peptide-based HIV protease inhibitors, DPM323 showed good oral bioavailability in animals,[174] which made it a good candidate for further development.

However, in phase I clinical trials, DPM323 (**47**) showed poor oral bioavailability in humans and highly variable blood levels (probably due to its low water solubility and high metabolism of the benzyl moiety), and the compound was withdrawn from phase I evaluation. Also the development of SC-52151 (**42**) has been terminated, because of lack of efficacy (based on CD4 cell counts and HIV RNA measurements). From the KNI series, KNI-272 (**48**) was selected for phase I clinical trials: it showed equal activity against HIV-1 as KNI-227 (**43**), but higher oral bioavailability. Another HIV protease inhibitor, that after extensive preclinical studies has entered phase I clinical testing is the non-peptidic, pyrone-based coumarin derivative U-96988 (**49**).[176] This molecule has excellent oral bioavailability in animals. Lowering the molecular weight may improve the oral bioavailability while maintaining high affinity for the HIV protease. This premise led to the development of VX-478 (**50**),[177] which is now also in phase I clinical trials.

Most advanced in clinical trials are saquinavir (Ro 31-8959), which has proved to provide additional benefit (i.e. in decreasing p24 levels and increasing CD4 cell counts) when combined with AZT and DDC (phase III clinical trials) and the two compounds [L-735,524 (also referred to as MK-639) (**51**)[178] and ABT-538 (**52**),[179] now both in phase II clinical trials] that have proved instrumental in monitoring the rapid turnover of plasma



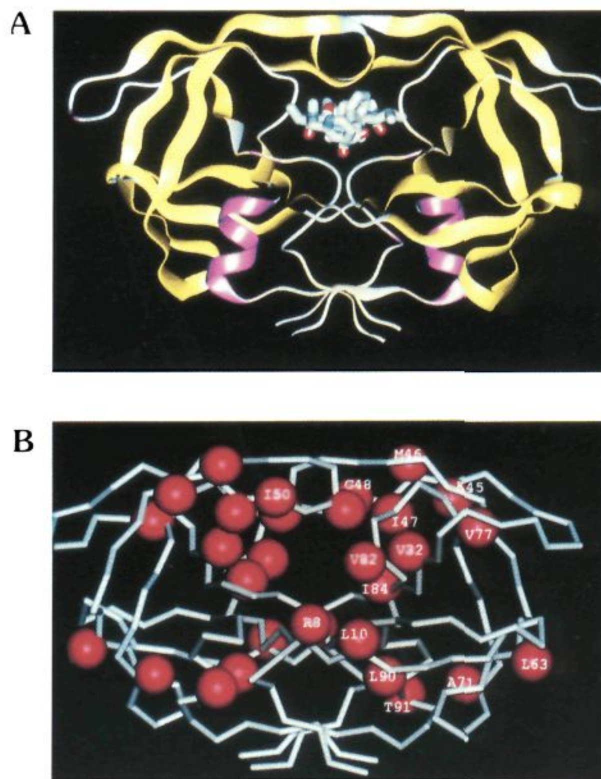


Figure 11. (A) Ribbon diagram of the C_2 backbone of HIV-1 protease complexed with the C_2 symmetry-based inhibitor, A-77003. Image rendered by R. Cachau and J. Erickson (NCI, FCRDC) based on the crystal structure.[185] (B) Backbone model of HIV-1 protease showing the position of residues where drug-resistant mutations have arisen under selection pressure of protease inhibitors *in vitro* and *in vivo* (T. N. Bhat and J. Erickson, NCI-FCRDC, unpublished).

virions in HIV-1-infected individuals.[36,37] Following oral treatment with L-735,524 or ABT-538, all patients showed an exponential decay of plasma viremia over the first two weeks, clearly attesting to the *in vivo* antiviral activity of these HIV protease inhibitors.

Although virus resistance to HIV protease inhibitors is believed to arise more slowly than with RT inhibitors,[180] HIV-1 resistance to the protease inhibitor Ro 31-8959 was obtained after only five passages of HIV-1 *in vitro* in the presence of the compound.[181] Resistance to C_2 symmetric inhibitors of HIV-1 protease has been described,[182] and is due to Val → Ala mutation at position 82 of the protease. Substitution of Gln or Lys for Arg at position 8 of the protease[183] leads to a marked resistance of HIV-1 to A-77003. Other mutations[105] that have been associated with HIV-1 resistance to HIV protease inhibitors are the following: 32 Val → Ile, 46 Met → Ile, 46 Met → Leu, 46 Met → Phe, and 82 Val → Ile (only if associated with 32 Val → Ile) for A-77003; 82 Val → Ala, 82 Val → Ile, 82 Val → Phe, 84 Ile → Val and 97 Leu → Val (only if associated with 82 Val → Ala) for DPM323;[184] and 48 Gly → Val and 90 Leu → Met for Ro 31-8959. The latter two mutations have been noted both *in vitro* and *in vivo*[105] and would, if combined, yield >100-fold resistance to Ro 31-8959. These mutations appear to be located in both the active and nonactive ("peripheral") site of the HIV protease (Figure 11). The "peripheral" mutations (i.e. 46 Met → Ile) do not appear to influence inhibitor association constants or the rate of synthetic substrate processing and their exact role in virus–drug resistance is not at all understood.

Myristoylation and Glycosylation Inhibitors

Formation of infectious virus particles not only depends on proteolytic cleavage of the *gag* and *gag-pol* precursor proteins by the HIV protease, but also on previous myristoylation (Figure10) of these precursor proteins. This myristoylation is carried out by a cellular enzyme, the protein *N*-myristoyltransferase which links myristic acid *via* an amide bond to the N-terminal glycine of Pr 55 and Pr 160. Several myristic acid derivatives, i.e. 13-oxatetradecanoic acid[186] and 12-azidododecanoic acid,[187] have been found to inhibit HIV-1 production in both acutely and chronically infected cells, but they only do so at relatively high concentrations (10–50 μM). This may not be therapeutically useful, as myristic acid derivatives may be expected to interfere with vital cellular processes (i.e. G protein-dependent signal transduction pathway) requiring myristoylation (i.e. α-subunit of G proteins).

The HIV envelope glycoproteins gp120 and gp41 have to undergo extensive glycosylation (Figure 12) to ensure infectivity of the HIV particles. These glycoproteins are involved in virus–cell binding and virus–cell fusion

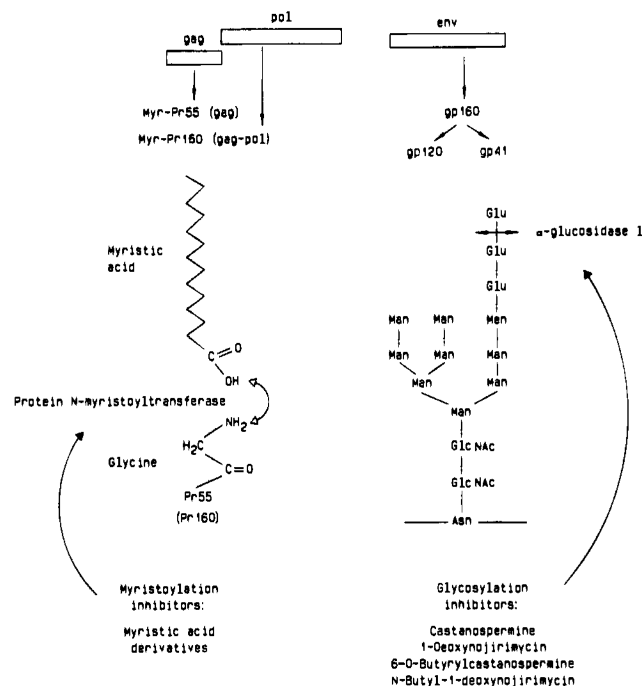
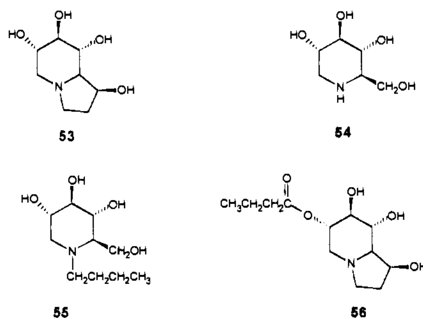


Figure 12. Myristoylation and glycosylation inhibitors are targeted at protein *N*-myristoyltransferase and α-glucosidase I, respectively. These enzymes are involved in the myristoylation of the *gag* and *gag-pol* precursor proteins and glycosylation of the envelope glycoproteins.

(*vide supra*), and thus glycosylation inhibitors may be assumed to decrease virus infectivity. This has indeed been shown for a number of compounds such as castanospermine (**53**), 1-deoxynojirimycin (**54**), *N*-butyl-1-deoxynojirimycin [SC-48334 (**55**)],[188] and 6-*O*-butanoylcastanospermine [MDL-28574 (**56**)].[189,190] Yet, these compounds inhibit virus infectivity only at rather high concentrations (0.1–10 mM). Their target enzyme should be α-glucosidase I, which is responsible for the cleavage of the terminal α-glucose unit. This enzyme initiates the trimming of the asparagine-linked oligosaccharides and thus is required to ensure formation of the mature glycans.



The anti-HIV activity of the glycosylation inhibitors may obviously be attributed to an altered glycosylation of the HIV envelope glycoproteins,[191] but how then could this aberrant glycosylation give rise to an attenuation of HIV infectivity? Several possibilities may account for this attenuated HIV infectivity: (i) abnormal folding of the nascent glycoproteins; (ii) diminished processing of the gp160 precursor glycoprotein to gp120 and gp41; and (iii) impaired processing of the gp120 to gp70 and gp50, which would normally happen through the aid of a trypsin-like protease once the gp120 glycoprotein has been docked to the CD4 receptor.[192]

Glycosylation inhibitors (i.e. *N*-butyl-1-deoxynojirimycin) are not only active against HIV but also other viruses (i.e. HBV) requiring glycosylation for the formation of infectious virus.[193] These compounds are neither very potent nor very selective in their anti-HIV action. Their selectivity must stem from quantitative differences between virus-infected and uninfected cells, in that the replicating virus puts higher demands on the glycosylation machinery than normal cell metabolism. As has been demonstrated particularly with 6-*O*-butanoylcastanospermine,[194] glycosylation inhibitors may, independently from their inhibitory effects on viral glycoprotein glycosylation, also interact with host cell adhesion molecules and thus prevent cell-to-cell spread of HIV.

Given the lack of specificity of the glycosylation inhibitors, and the fact that they are essentially targeted at a host cell process, it is not surprising that HIV resistance to these compounds has not been reported (although different HIV strains may vary markedly in their sensitivity to castanospermine).[46] Castanospermine has proven effective against murine leukemia virus infection *in vivo*, but, when compared to AZT, it was less active and more toxic.[195] Due to their inhibitory effect on α-glucosidases (including intestinal α-glucosidases), glycosylation inhibitors may be expected to lead to gastrointestinal discomfort (i.e. diarrhea). This problem might be overcome by prodrugs (i.e. the 6-phosphate derivative of *N*-butyl-1-deoxynojirimycin)[192] which do not inhibit gut α-glucosidases. To fulfil their goal, these prodrugs should be able to pass as such the intestinal barrier before they are hydrolyzed to release the active compound.

Combination Therapy

It is now taken for granted that as for the chemotherapy of a variety of bacterial and malignant diseases, the treatment of AIDS will eventually be based on the combination of two, three, or even more anti-HIV agents. The term combination is often understood as the simultaneous use of two or more drugs. Three "virtues" are generally associated with the combined use of different anti-HIV drugs: (i) diminished toxicity, due to a reduction in the dosage of the individual compound; (ii) reduced risk of virus–drug resistance development; and (iii) synergistic antiviral activity. These premises may indeed be fulfilled if the individual compounds that have to be combined (i) do not have overlapping toxicity profiles, (ii) lead to different, and preferably mutually antagonizing (see: *infra*) resistance mutations in the viral genome, and (iii) are targeted at different viral proteins, or within the same viral protein at different molecular sites.

In combining different anti-HIV drug, three different types of combinations could be envisaged. *First-level* drug combinations would consist of drugs that interact with different viral proteins (or enzymes), i.e. inhibitors of virus adsorption, virus–cell fusion, and/or uncoating combined with reverse transcriptase (RT) inhibitors combined with viral protease inhibitors. *Second-level* drug combinations would comprise those compounds that interact with different target sites within the same viral protein (enzyme), thus, among the RT inhibitors, the ddN analogues that are targeted at the substrate binding site combined with the NNRTIs that are

targeted at a nonsubstrate binding site. *Third-level* drug combinations could then be conceived as combinations of those drugs that interact with the same target site of the same protein (enzyme) but lead to mutually antagonistic or suppressive resistance mutations, as has been observed among certain ddN analogues (i.e. AZT *versus* DDI, DDC, or 3TC) as well as among the NNRTIs (i.e. BHAP *versus* TIBO, nevirapine, or pyridinone).[104] In particular, the combination of AZT with 3TC has proved to effect a more pronounced and sustained suppression of viral load, accompanied by a sustained increase of CD4 cells, than observed with AZT alone. Concomitantly, 3TC has been found to suppress emergence of the AZT-resistance mutation (215 Thr → Tyr) (*vide infra*).

To cite only examples of those combinations that contained AZT and that were found to act synergistically, at least in cell culture: *first-level* combinations of AZT with the glycosylation inhibitor castanospermine,[196] or with the protease inhibitor Ro 31-8959,[197] or with the Tat inhibitor Ro 24-7429;[198] *second-level* combinations of AZT with the NNRTIs HEPT,[199] I-EBU (MKC-442),[200] nevirapine,[201] TIBO,[202] or BHAP;[203] and *third-level* combinations of AZT with other ddN analogues such as PMEA[204] or DDI.[205,206] The effectiveness of these two-drug combinations can be further enhanced if extended to three-drug regimens, i.e. AZT combined with I-EBU and 6-*O*-butanoylcastanospermine (MDL-28,574),[200] or AZT combined with DDI and nevirapine,[207] or even four-drug regimens; as a rule, it can be stated that multidrug regimens are more effective in inhibiting HIV-1 replication than single-drug regimens and that the effectiveness increases with increasing the number of drugs in the combination.[208]

The combination of DDI with ribavirin deserves a special quotation because it is based on a different strategy than those outlined above. Ribavirin potentiates the anti-HIV activity of DDI (and other purine ddN analogues)[209,210] through interference with the metabolic pathway of these compounds and their cellular counterparts. Ribavirin is a well-known inhibitor of IMP dehydrogenase, which is needed to convert IMP to XMP that is then further converted to GMP, GDP, and GTP. On the one hand, ribavirin will increase the intracellular pool levels of IMP, thereby facilitating the conversion of DDI to ddIMP (which will then be converted to the antivirally active metabolite ddATP), since IMP is used as phosphate donor by 5′-nucleotidase to convert DDI to ddIMP.[211] On the other hand, ribavirin causes a depletion of the GTP pools, and as GTP serves as an obligatory cofactor in the conversion IMP to succinyl AMP, which is then further converted to AMP, ADP, ATP, and *via* ADP → dADP, to dATP, ribavirin decreases the pool levels of dATP, the direct competitor for ddATP at the HIV RT level. Thus, ribavirin potentiates the anti-HIV activity of DDI by a dual mechanism: enhancing the formation of its active metabolite ddATP and decreasing the formation of its competing natural substrate dATP.[212]

HIV Drug Resistance

The potential of HIV to develop resistance to anti-HIV drugs has led to an increasing concern, ever since it was reported that HIV variants isolated from patients following prolonged AZT therapy show reduced sensitivity to the drug.[213] The mutations 41 Met → Leu, 67 Asp → Asn, 70 Lys → Arg, 215 Thr → Phe/Tyr, and 219 Lys → Gln in the HIV-1 RT were found to contribute to high-level resistance of the virus to AZT.[214,215] The 215 Thr → Tyr mutation has been most frequently detected among AZT-resistant HIV-1 isolates from patients under prolonged AZT therapy.[216] The 74 Leu → Val mutation in HIV-1 RT is responsible for resistance to DDI,[217] the mutation 184 Met → Val for resistance to 3TC and DDC,[218,219] and the mutations 69 Thr → Asp and 65 Lys → Arg for resistance to DDC, 3TC, and DDI,[220-222] and the mutation 75 Val → Thr for resistance to D4T.[223] The patterns of HIV resistance, as emerging, to both inhibitors of HIV-1 reverse transcriptase and protease are summarized in Table 1.

HIV drug resistance may arise when single mutations which do not diminish viral replicative ability overcome the selective pressure of the drug at its trough concentration. The NNRTIs have been found to rapidly lead to the emergence of drug-resistant variants both *in vitro* (cell culture) and *in vivo* (patients).[99,104] The mutation 181 Tyr → Cys is associated with resistance (or reduced sensitivity) to most of the NNRTIs (i.e. TIBO, HEPT, nevirapine, pyridinone, BHAP, TSAO, α-APA), the mutation 188 Tyr → His is associated with resistance to TIBO and pyridinone but not nevirapine, the mutation 188 Tyr → Cys is associated with resistance to TIBO, pyridinone and nevirapine, the mutation 100 Leu → Ile is associated mainly with resistance to TIBO, the mutation 103 Lys → Asn is associated with resistance to TIBO, nevirapine, pyridinone, and BHAP, the mutation 106 Val → Ala is associated mainly with resistance to nevirapine, the mutation 108 Val → Ile is associated with resistance to nevirapine and pyridinone, the mutation 138 Glu → Lys accounts for resistance to TSAO (but not any of the other compounds), the mutation 190 Gly → Glu is responsible for resistance to quinoxaline S-2720, and the mutation 236 Pro → Leu leads to resistance to BHAP (but not any of the other NNRTIs; see Table 1).

Whereas the mutations conferring resistance to the ddN analogues are located in the vicinity of either the substrate (dNTP) binding site or template binding site (Figure 4), the mutations conferring resistance to the NNRTIs tend to cluster around the putative binding site of these compounds (Figure 5). The ddN mutations do not lead to cross-resistance to the NNRTIs, and, *vice versa*, NNRTI mutations do not lead to cross-resistance to the ddNs; within each group, the compounds do not necessarily give cross-resistance. Although not rigorously corroborated by comparative studies, resistance to the ddN analogues may develop slower than for the NNRTIs, and as shown particularly for AZT, resistance may develop in a stepwise fashion, through the accumulation of multiple mutations, each step leading to accruing resistance.[226]

Resistance development has also been noted for the HIV protease inhibitors,[181-183] another class of highly specific anti-HIV agents. The mutations conferring resistance to the protease inhibitors have been identified (Table 1). As for the NNRTIs, it can be assumed that these mutations directly affect the binding of the inhibitors to their target molecule, whether the reverse transcriptase or protease.

The clinical relevance of virus–drug resistance, *viz.*

Table 1. Patterns of HIV Drug Resistance for Reverse Transcriptase Inhibitors and Protease Inhibitors[a]

1. HIV-1 Reverse Transcriptase: Substrate Binding Site

	inhibitor				
mutation	AZT	DDI	DDC	D4T	3TC
41 Met → Leu	+				
50 Ile → Thr				+	
65 Lys → Arg			+		
67 Asp → Asn	+				
69 Thr → Asp			+		
70 Lys → Arg	+				
74 Leu → Val		+	+		
75 Val → Thr		+	+	+	
184 Met → Val		+	+		+
215 Tyr → Cys			+		
215 Thr → Tyr/Phe	+				
219 Lys → Gln/Glu	+				

2. HIV-1 Reverse Transcriptase: Nonsubstrate Binding Site

	inhibitor							
mutation	TIBO R82913	HEPT MKC-442	Nevirapine	Pyridinone L-697661	BHAP U-87201	TSAO	α-APA	quinoxaline S-2720
98 Ala → Gly			+	+				
100 Leu → Ile	+		+	+	+			
101 Lys → Glu				+	+			
103 Lys → Asn	+		+	+	+			
106 Val → Ala	+	+	+		+	+	+	+
108 Val → Ile		+	+	+				
138 Glu → Lys						+		
179 Val → Asp/Glu	+			+				
181 Tyr → Cys	+	+	+	+		+	+	+
181 Cys → Ile	+		+	+	+	+		+
188 Tyr → Cys	+		+	+				
188 Tyr → His	+	+		+	+			
190 Gly → Glu/Ala	+		+	+	+			+
236 Pro → Leu					+			

3. HIV-1 Protease

	inhibitor						
mutation	Ro 31-8959 (Saquinavir)	A-77003	ABT-538	MK-639 (L735524)	KNI-272	SC-52151	DPM323
8 Arg → Gln/Lys		+					
10 Leu → Phe							+
24 Leu → Val						+	
32 Val → Ile		+		+	+		
45 Lys → Ile							+
46 Met → Ile/Leu/Phe		+		+			
47 Ile → Leu				+			
48 Gly → Val	+	+		+		+	+
63 Leu → Pro				+			
71 Ala → Val				+		+	
82 Val → Ala		+		+		+	+
82 Val → Ile		+					+
82 Val → Phe			+	+			+
84 Ile → Val	+		+	+	+		+
90 Leu → Met	+	+		+			

[a] The data were extracted from refs 99, 104, 105, 224, and 225.

its role in disease progression, remains to be determined. It is generally felt that emergence of resistance to a given drug limits, or even argues against, the clinical usefulness of the compound. Yet, drug-resistant virus variants may be less pathogenic than the wild-type variants. Otherwise, they should not be overgrown by the wild-type in the absence of any selective drug pressure. In a clinical study with AZT-treated patients, the presence of syncytium-inducing HIV-1 strains (which are thought to be more pathogenic than the non-syncytium-inducing HIV-1 strains) and the RT codon 215 mutation (imparting resistance to AZT) were found to correlate with a high virus burden and marked decline of the CD4 cell counts (which can be considered as a marker of disease progression).[227] However, it was not clarified from this study whether the codon 215 mutation gave rise to syncytium induction, or, *vice versa*, whether syncytium induction gave rise to the codon 215 mutation, or, whether both, interdependently or independently led to disease progression.

If less pathogenic than the wild-type, drug-resistant virus strains may also be less readily transmitted from one person to another. There is anecdotal evidence for the transmission of AZT-resistant HIV-1 variants through either homosexual contact[228] or heterosexual contact[229] or unrecognized exposure to blood.[230] However, when the sexual transmission of AZT-resistant HIV-1 variants (containing the 215 Thr → Tyr mutation) was investigated in donor-recipient pairs,[231] AZT-resistant virus was found in only one of the four recipients, which points to an apparent selection against transmission of AZT-resistant HIV-1 variants.

The reversibility of the drug-resistant phenotype, and the underlying mutation(s), is another issue that should be followed up. AZT-resistant HIV-1 mutants may persist for a long time (1 year or even longer) after cessation of AZT therapy, as is suggested by several studies.[232-236] This may not be surprising in view of high-level AZT resistance being based on the accumulation of multiple mutations in the RT genome. It has not been determined how long it takes for NNRTI-resistant HIV-1 mutants to revert to the wild-type. For pyridinone L-697,661, resistance *in vivo* in the patient develops within 12 weeks of treatment,[237] but it is not known whether and after how much time the NNRTI-resistant phenotype reverted to the wild-type. As HIV-1 resistance to NNRTIs generally depends on one mutation, the time required for the mutant to the wild-type, following withdrawal of the drug, may not be as long as for the AZT-resistant HIV-1 variants.

Provided that virus drug-resistance development indeed compromises the clinical outcome of drug therapy, what measures could be taken to circumvent or prevent resistance development? If resistance develops to one of the NNRTIs, treatment could be switched to any of the other NNRTIs to which the virus has retained sensitivity. For example, TSAO-resistant HIV-1 mutant strains, containing the 138 Glu → Lys mutation can be completely suppressed by any other HIV-1-specific RT inhibitor (i.e. TIBO, nevirapine, BHAP, etc.).[238] 5-Chloro-3-(phenylsulfonyl)indole-2-carboxamide retains activity against those HIV-1 strains that, because of the 103 Lys → Asn or 181 Tyr → Cys mutation, have acquired resistance to other NNRTIs (i.e. TIBO, nevirapine, pyridinone).[239] The α-APA derivative R89439 is very active against the 100 Leu → Ile mutant, which is highly resistant to the TIBO derivatives.[240] As already mentioned,[118] shifting the chlorine atom from the 9-position (as in R82913) to the 8-position (as in R86183) restores the activity against the 181 Tyr → Cys mutant.

Similarly, pyridinone L-702019, which differs from its predecessor L-696,229 only by the addition of two chlorine atoms (in the benzene ring) and substitution of sulfur for oxygen (in the pyridine ring), retains activity against HIV-1 mutants containing the 103 Lys → Asn or 181 Tyr → Cys mutation.[241] In some instances, resistance to one of the NNRTIs may even be accompanied by hypersensitivity to others, i.e. the 236 Pro → Leu mutation causing resistance to BHAP confers a 10-fold increased sensitivity to TIBO, nevirapine, and pyridinone.[242] Under the continuous pressure of the compounds the 181 Tyr → Cys mutation further shift to the 181 Cys → Ile mutation, and this generates high-level resistance to most of the NNRTIs, except for the HEPT derivatives which retain marked activity against this mutant.[238]

While therapy could be switched from compound A to compound B, when HIV develops resistance to compound A, these compounds could also be combined from the beginning, since several compounds appear to give rise to mutually antagonistic mutations. In this sense, the 236 Pro-Leu mutation responsible for BHAP resistance partially restores the sensitivity of the 181 Tyr → Cys mutants to TIBO, nevirapine, and pyridinone.[242] The 181 Tyr → Cys mutation, which causes resistance to most of the NNRTIs, has been found to suppress the 215 Thr → Tyr mutation causing resistance to AZT,[243] and, *vice versa*, the 181 Tyr → Cys mutation can be suppressed by AZT,[244] which thus means that the NNRTI mutation at position 181 and the AZT mutation at position 215 of the HIV-1 RT seem to antagonize each other. Yet other mutations have proved to counteract each other:[104] i.e. 236 Pro → Leu *versus* 138 Glu → Lys; 215 Thr → Tyr/Phe *versus* 184 Met → Val; and 215 Thr → Tyr/Phe *versus* 74 Leu → Val. On the basis of the mutations that are mutually suppressive, different drugs could be rationally chosen for multidrug regimens so as to suppress development of resistance to one another. In particular, the AZT-resistance mutation at position 215 is counteracted by the 3TC-resistance mutation at position 184 and the NNRTI (i.e. α-APA)-resistance mutation at position 181, and this provides a sufficient rationale for the dual combination of AZT with 3TC, and the triple combination of AZT (zidovudine) with 3TC (lamivudine) and α-APA (loviride).

In the clinic,[244] in HIV-1-infected patients, concomitant therapy of nevirapine with AZT was found to suppress the emergence of the most common NNRTI mutation (namely 181 Tyr → Cys), although other mutations (i.e. at the RT positions 188 and 190) emerged under the selective pressure of the drugs. The latter may not be surprising if under the conditions used virus replication was not totally suppressed. In view of its highly dynamic replicative ability,[35-37] the virus may readily escape from any treatment regimen, if it were not completely "knocked out" from the beginning.

This would then bring us to what might seem a particularly attractive approach to prevent the emergence of drug-resistant HIV variants, that is the use, from the start, of "knocking-out" drug concentrations.[119,120] If, as already discussed above, the NNRTIs, i.e. TIBO, HEPT, nevirapine, pyridinone, or BHAP, are added to the HIV-1-infected cell cultures at a sufficiently high concentration (well below the cytotoxicity threshold), they can apparently "sterilize" the cell culture from the virus infection and prevent the breakthrough of any virus, whether resistant or not. This "knocking-out" phenomenon has been observed with the NNRTIs[119,120] and also with the HIV protease inhibitors, but not with the ddN analogues (i.e. AZT).[120,121]

With the HIV-1-specific RT inhibitor quinoxaline S-2720, a complete clearance of virus from the virus-infected cell cultures could be achieved at a concentration (as low as 0.35 μM) that could be readily achieved therapeutically *in vivo* upon systemic administration of the drug to patients.[245] With the HIV protease inhibitor Ro 31-8959 at a concentration of 0.1 μM, a complete "cure" of HIV-1(III_B) infection in MT-4 cells was achieved upon a 3-month treatment period.[246] Thus NNRTIs as well as HIV protease inhibitors can achieve an apparent clearance of HIV from the cell cultures if used from the beginning at a sufficiently high concentration. This "knocking-out" effect on the virus could probably be accomplished at lower concentrations if the individual compounds were to be combined. Thus an advisable strategy to be pursued in the future may be based on the use of drug combinations containing one or more NNRTIs and one or more HIV protease inhibitors. Such drug combinations may completely block virus replication, prevent virus–drug resistance and eventually clear the cells from the infection.

Long-term survival of persons that have remained symptom-free for many years despite HIV-1 infection seems to be associated with a low, although persisting, viral load.[247,248] This should guide us in our therapeutic efforts against AIDS, as any therapy should aim at reducing the burden of HIV-1 to the levels seen in long-term survivors or below. Ideally, the virus burden should be reduced to such an extent that it is no longer detectable. This should not be too far fetched a goal if, as it has recently been reported for a perinatally infected infant,[249] HIV infection can indeed be cleared from the body.

Future Directions

Now, one decade after the perspectives for the chemotherapy of HIV infections were discussed for the first time,[250] it cannot be denied that the originally formulated premises have been largely fulfilled. We now have at hand a multitude of potentially therapeutic agents targeted at virtually all stages of the HIV replicative cycle. Some of the HIV inhibitors are targeted at an early viral event (i.e. virus adsorption, fusion, or uncoating), whereas others are targeted at a late viral event (i.e. proteolytic cleavage of the viral precursor proteins). But the majority of the HIV inhibitors are directed toward the reverse transcriptase (RT), which has thus remained the most attractive chemotherapeutic target ever since the causative agent of AIDS was proved to be a retrovirus. Among the inhibitors of viral fusion/uncoating (i.e. bicyclam derivatives), reverse transcriptase (i.e. HEPT and α-APA derivatives), and HIV protease, some representative congeners have been found to inhibit HIV replication at nanomolar concentrations that are about 100 000-fold below the cytotoxicity threshold. Such selectivity is unprecedented in that it has never before been achieved with any other antiviral agents. Yet, a "cure" for AIDS in the sense of a drug that would definitively clear HIV from the patient has not been accomplished. Also, the armamentarium of formally approved anti-HIV drugs is not particularly impressive, not quantitatively (only four compounds) and, even less so, qualitatively (all four being ddN analogues and thus related to each other in chemical structure and mode of action). The diversity of new compounds now available and known to act at a variety of molecular targets within the viral replicative cycle should allow a more diverse choice for drug licensing in the near future.

Why are we still in abeyance of a "cure" for AIDS? One of the major problems compromising a definitive "cure" for AIDS is the propensity of the virus to continuously mutate and hence escape the inhibitory effects of the more specific anti-HIV agents. It can be postulated that the more specific the compound for HIV, the easier it leads to drug resistance, and thus emergence of drug resistance has been noted particularly with the highly HIV-1-specific non-nucleoside RT inhibitors. Yet, resistance should not be a cause for despair. Strategies should be pursued to prevent or circumvent resistance. One of these strategies may be based on the combination of different drugs that interact with different molecular targets, or, if interacting with the same target, lead to mutually antagonistic resistance mutations. Another attractive strategy would be based on using sufficiently high drug doses from the beginning so as to completely "knock out" the virus. Both strategies should, ideally, be combined, so as to knock out the virus with lower concentrations of the drugs combined than when used individually. With such drug combinations containing the appropriate compounds at the appropriate concentrations, it has proven possible to completely suppress virus replication in cell culture, and to prevent virus (whether drug-resistant or not) from breaking through. If this "knock-out" proposal, even if seemingly optimistic, could be extrapolated to the *in vivo* situation, it should be feasible to tipping the balance toward virus elimination and recovery from the disease.

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Biography

Erik De Clercq received his M.D. degree in 1966 and his Ph.D. in 1972, both from the Katholieke Universiteit Leuven in Belgium. After postdoctoral training at Stanford University first as a Lilly International Fellow and subsequently as a Damon Runyon Cancer Research Fellow. Dr. De Clercq returned to Leuven University Medical School where he became Professor in 1975. He served as Chairman of the Department of Microbiology from 1986 until 1991. In 1986 he also became Chairman of the Directory Board of the Rega Institute. In 1994, Dr. De Clercq was elected a Fellow of the American Association for the Advancement of Science and in 1995 was awarded the Professor P. De Somer Chair for Microbiology of the Katholieke Universiteit Leuven.

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