Current Medicinal Chemistry
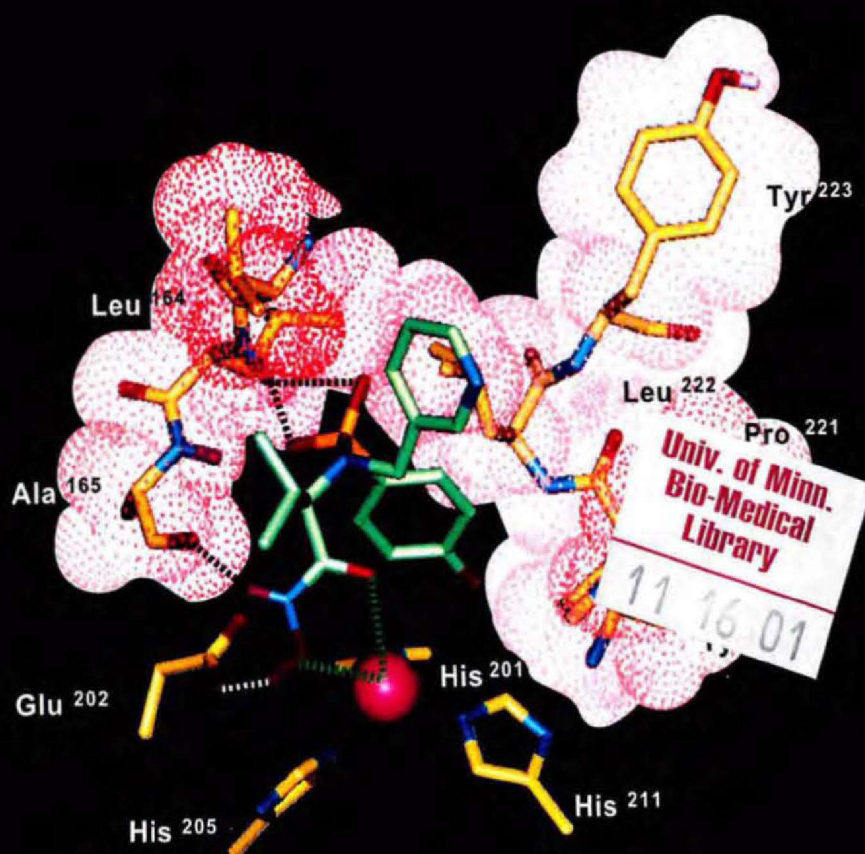
The International Journal for Timely In-Depth Reviews in Medicinal Chemistry

New Developments in Anti-HIV Chemotherapy

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Abstract: Virtually all the compounds that are currently used, or under advanced clinical trial, for the treatment of HIV infections, belong to one of the following classes: (i) nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs): i.e., zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC), emtricitabine [(-)FTC], tenofovir (PMPA) disoproxil fumarate; (ii) non-nucleoside reverse transcriptase inhibitors (NNRTIs): i.e., nevirapine, delavirdine, efavirenz, emivirine (MKC-442); and (iii) protease inhibitors (PIs): i.e., saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, and lopinavir. In addition to the reverse transcriptase and protease step, various other events in the HIV replicative cycle are potential targets for chemotherapeutic intervention: (i) viral adsorption, through binding to the viral envelope glycoprotein gp120 (polysulfates, polysulfonates, polyoxometalates, zintevir, negatively charged albumins, cosalane analogues); (ii) viral entry, through blockade of the viral coreceptors CXCR4 and CCR5 [bicyclams (i.e. AMD3100), polyphemusins (T22), TAK-779, MIP-1α LD78β isoform]; (iii) virus-cell fusion, through binding to the viral glycoprotein gp41 [T-20 (DP-178), T-1249 (DP-107), siamycins, betulinic acid derivatives]; (iv) viral assembly and disassembly, through NCp7 zinc finger-targeted agents [2,2'-dithiobisbenzamides (DIBAs), azadicarbonamide (ADA) and NCp7 peptide mimics]; (v) proviral DNA integration, through integrase inhibitors such as L-chicoric acid and diketo acids (i.e. L-731,988); (vi) viral mRNA transcription, through inhibitors of the transcription (transactivation) process (fluoroquinolone K-12, *Streptomyces* product EM2487, temacrazine, CGP64222). Also, in recent years new NRTIs, NNRTIs and PIs have been developed that possess respectively improved metabolic characteristics (i.e. phosphoramidate and *cyclo*saligenyl pronucleotides of d4T), or increased activity against NNRTI-resistant HIV strains [second generation NNRTIs, such as capravirine and the novel quinoxaline, quinazolinone, phenylethylthiazolylthiourea (PETT) and emivirine (MKC-442) analogues], or, as in the case of PIs, a different, non-peptidic scaffold [i.e. cyclic urea (DMP 450), 4-hydroxy-2-pyrone (tipranavir)]. Given the multitude of molecular targets with which anti-HIV agents can interact, one should be cautious in extrapolating from cell-free enzymatic assays to the mode of action of these agents in intact cells. A number of compounds (i.e. zintevir and L-chicoric acid, on the one hand; and CGP64222 on the other hand) have recently been found to interact with virus-cell binding and viral entry in contrast to their proposed modes of action targeted at the integrase and transactivation process, respectively.

INTRODUCTION

Combination therapy, comprising at least three anti-HIV drugs, has become the standard treatment of AIDS or HIV-infected patients. Virtually all drugs that have been licensed for clinical use (or made available through expanded access programmes) for the treatment of HIV infections fall into one of the following three categories: (i), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), that, following two phosphorylation steps (tenofovir) or three phosphorylation steps (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir), act, as chain terminators, at the substrate binding site of the reverse transcriptase; (ii), non-nucleoside reverse transcriptase inhibitors (NNRTIs), that interact with the reverse transcriptase at an allosteric, non-substrate binding site (nevirapine, delavirdine, efavirenz);

and (iii), protease inhibitors (PIs), that specifically inhibit, as peptidomimetics, the virus-associated protease (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir). Guidelines to the major clinical trials with these compounds have been recently published [1].

In numerous studies, combinations of NRTIs, NNRTIs and PIs have been found to decrease HIV viral load, increase CD4 count, decrease mortality and delay disease progression, particularly in AIDS patients with advanced immune suppression [2]. When initiated during early asymptomatic HIV infection, highly active antiretroviral (combination) therapy (HAART) initiates rapid reversal of disease-induced T-cell activation, while preserving pretherapy levels of immune function, suggesting that therapeutic benefit may be gained from early aggressive anti-HIV chemotherapy [3]. Combination therapy that produces sustained suppression of plasma HIV RNA may also be able to reduce the virus burden in the lymphoid tissues [4], although clearance of plasma viremia is not invariably associated with immune restoration [5] and not at all

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paralleled by a reduction in viral DNA burden in the peripheral blood mononuclear cells [6].

The introduction of HAART or combined anti-HIV drug regimens has had profound repercussions on various AIDS-associated diseases. While partial immune restitution induced by HAART in patients with advanced HIV infection can exacerbate clinically apparent cryptococcal meningitis [7] or CMV vitritis, HAART may reduce the prevalence of cervical squamous intra-epithelial lesions in HIV-seropositive women caused by HPV [8]. Several studies have indicated that HAART significantly improves the
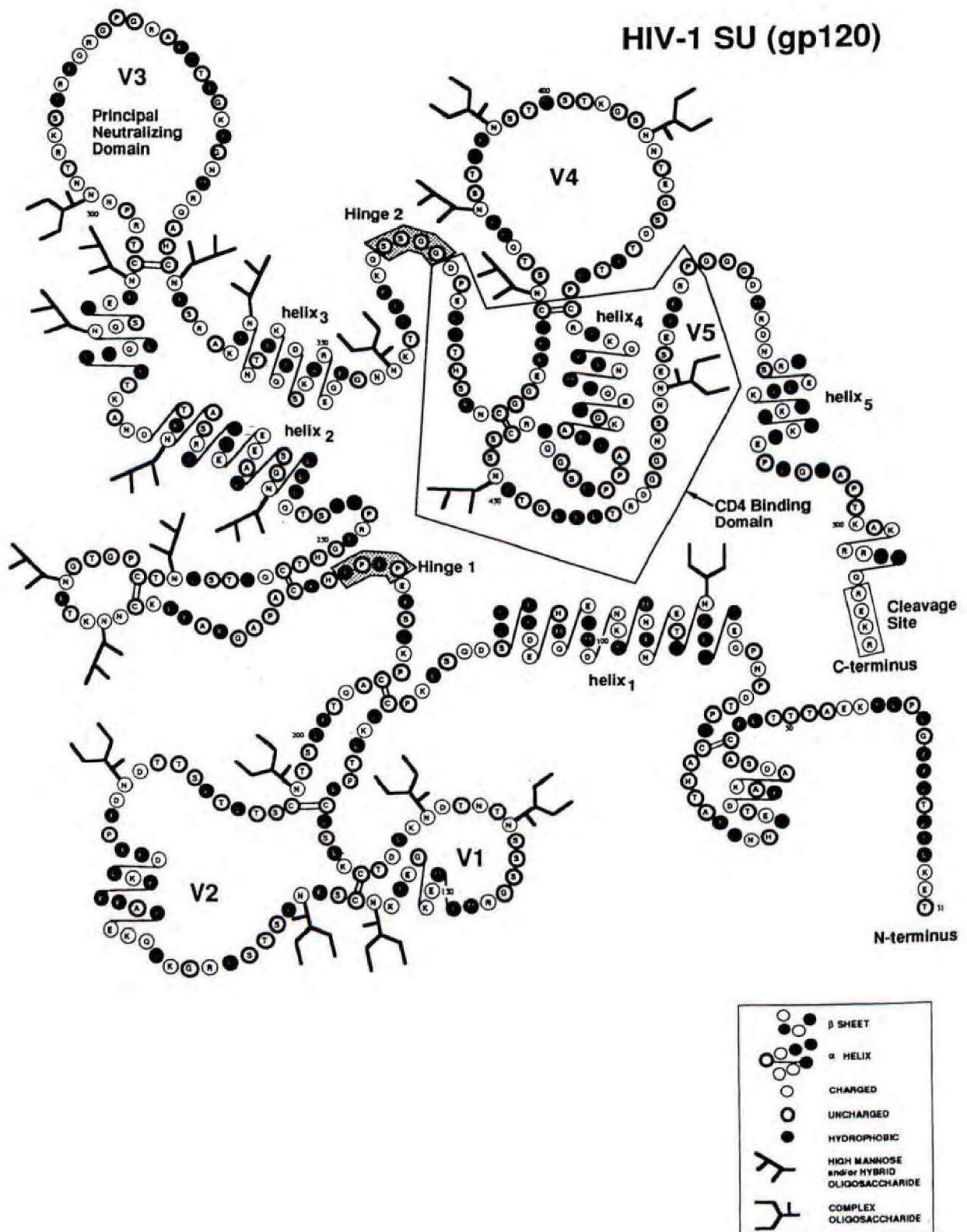

Fig. (1). Model for the surface (SU) glycoprotein gp120. Figure taken from ref. 22.

prognosis of AIDS patients with progressive multifocal leukoencephalopathy [9-11]. Likewise, HAART has been shown to favorably alter the prognosis of CMV retinitis in HIV-infected individuals, as attested by long-lasting remission of CMV retinitis without CMV maintenance therapy [12], lack of reactivation of CMV retinitis after stopping CMV maintenance therapy [13], decrease of CMV replication (viremia) [14,15] and increased survival [16,17]. In the era of combination antiretroviral therapy the management of CMV diseases in patients with AIDS has undergone dramatic modifications [18,19].

Although the long-term goal of eradicating the virus from latently and chronically infected cells remains forbidding [20] the advent of so many new compounds, other than those that have been formally approved, for the treatment of HIV infections, will undoubtedly improve the prognosis of patients with AIDS and AIDS-associated diseases. Here I will primarily address those new anti-HIV compounds that (i) have emerged as promising anti-HIV drug candidates during the last few years, that (ii) are in preclinical or early-clinical development, and that (iii) are targeted at well-defined steps in the HIV replicative cycle.

VIRUS ADSORPTION (gp120) INHIBITORS

A great variety of polyanionic compounds have been described to block HIV replication through interference with virus adsorption (or binding) to the cell surface: i.e., polysulfates, polysulfonates, polycarboxylates, polyphosphates, polyphosphonates, polyoxometalates, etc. This class of compounds also comprises the sulfated polysaccharides extracted from sea algae [21]. All these compounds whether synthetic or of natural origin, are assumed to exert their anti-HIV activity by shielding off the positively charged sites in the V3 loop of the viral envelope glycoprotein (gp120) [22] which is necessary for virus attachment to the cell surface heparan sulfate, a primary binding site, before more specific binding occurs to the CD4 receptor of the CD4$^+$ cells (Fig. 1). Heparan sulfate is widely expressed on animal cells and, as it is involved in the virus-cell binding of a broad spectrum of enveloped viruses, including HSV [23], it also explains why polysulfates have a broad-spectrum antiviral activity against HIV, HSV and various other enveloped viruses.

The major role of polysulfates or polyanionic substances in general in the management of HIV infections may reside in the prevention of sexual transmission of HIV infection, as these compounds, if applied as a vaginal formulation, may successfully block HIV infection through both virus-to-cell and cell-to-cell contact. These compounds therefore merit being pursued as vaginal microbicides. The fact that in addition to their anti-HIV activity, these polyanionic substances also inhibit other sexually transmitted disease (STD) pathogens further adds to their potential therapeutic and preventive value. One candidate compound is poly(sodium (4-styrene sulfonate) which is highly effective against several STD pathogens including not only HSV but also *Neisseria gonorrhoeae* and *Chlamydia trachomatis* [24].

Foremost among the polyanionic substances that have been described as virus-cell binding inhibitors are dextran sulfate, dextrin sulfate, polyvinylalcohol sulfate (PVAS) (Fig. 2), polyacrylic acid/polyvinylalcohol sulfate copolymer (PAVAS) (Fig. 2) (and many other polysulfates), naphthalene sulfonate (PRO2000) (Fig. 2) [25], polyoxometalates such as JM1590 or $K_{13}[Ce(SiW_{11}O_{39})_2].26\ H_2O$ (Fig. 2) and JM2766 or $K_6[BGa(H_2O)W_{11}O_{39}].15\ H_2O$ [26], and the negatively charged (i.e., succinylated) human serum albumins [28]. Polyoxometalates can assume various structures, such as Keggin, Dawson, "Double" Keggin, "Double" Dawson, "Triple" Keggin and "Large" Ring structures: they all inhibit virus adsorption through interference with the binding of the viral envelope glycoprotein gp120 to CD4$^+$ cells [27].

Zintevir (AR177, T30177), which is capable of forming a double guanosine quartet (G-quartet) [29-31], was originally described as an inhibitor of HIV-1 integrase (see *infra*). Yet, its primary target, accounting for its anti-HIV activity in cell culture, does not appear to be the integrase, but the viral adsorption step [32,33] As first described for dextran sulfate [34], resistance to polyanionic substances can arise through repeated passage of HIV-1 in the presence of the compound, and this resistance is invariably mediated by mutations in the gp120 molecule, i.e., S114N (V1 loop), S134N (V2 loop), K269E, Q278H, N293D (V3 loop), N323S (C3 region), deletion of FNSTW at positions 364-368 (V4 loop) and R387I (CD4 binding domain). It was later shown that HIV-1 resistance to other polyanionic substances, that are targeted at the virus adsorption step, such as zintevir [33], cyclodextrin sulfate [35] and negatively charged albumins [36], emanates through a similar set of mutations in gp120. The viral gp120 glycoprotein must thus be considered as the main target for the anti-HIV-1 action of zintevir and the other polyanionic substances.

Cosalane analogues represent another class of molecules that owe their anti-HIV activity to the polyanionic cosalane pharmacophore [37-39]. Cosalane analogues such as that shown in Fig. 2 are able to bind to either gp120 or CD4 or both, thus affecting the interaction of gp120 with CD4, and the ensuing virus-cell attachment and fusion processes [37-39]. A hypothetical model has been proposed for the binding of the cosalane motif with CD4, which should assist in the design of more efficient congeners [38].

VIRAL CORECEPTOR ANTAGONISTS

To enter cells, following binding with the CD4 receptor, the HIV-1 particles must interact, again through the viral envelope glycoprotein gp120, with the CXCR4 coreceptor [40] or CCR5 coreceptor [41] (Fig. 3). CXCR4 is the coreceptor for HIV-1 strains that infect T-cells (T-tropic or X4 strains), and CCR5 is the coreceptor for HIV-1 strains that infect macrophages (M-tropic or R5 strains). CXCR4 and CCR5 have not evolved simply to act as coreceptors for HIV entry; they normally act as receptors for chemokines (chemoattractant cytokines). The normal ligands for CCR5 are RANTES ("regulated upon activation, normal T-cell expressed and secreted") and MIP-1α and -1β ("macrophage
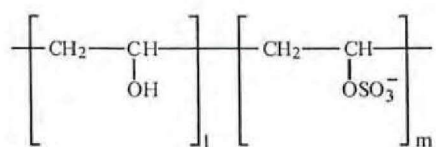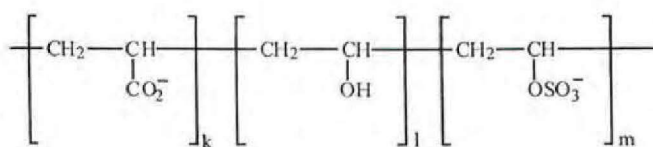
PVAS

PAVAS
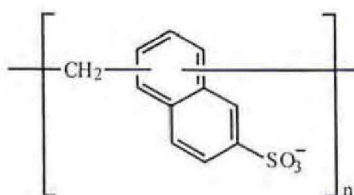
Naphthalene sulfonate
PRO 2000
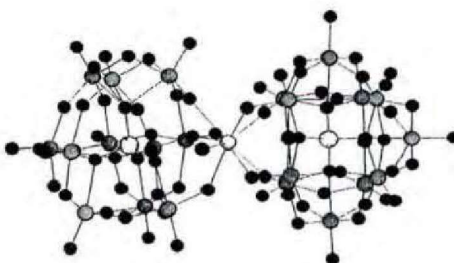
$K_{13}[Ce(SiW_{11}O_{39})_2].26\ H_2O$
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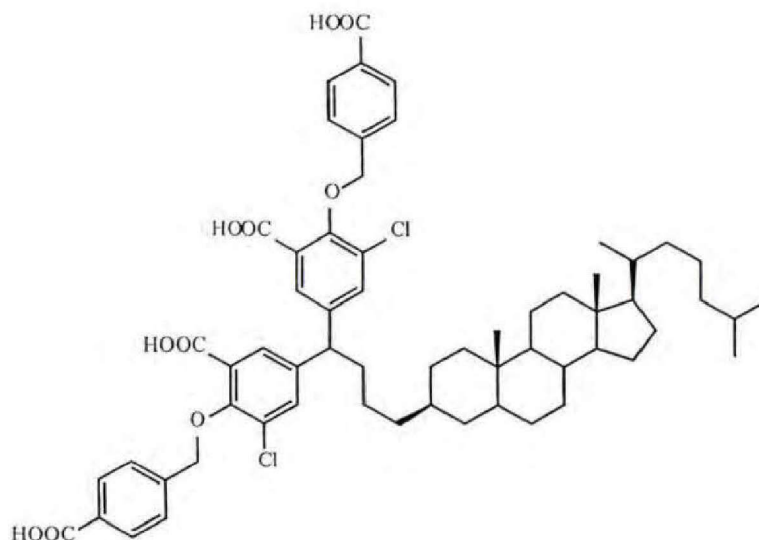
Cosalane analogue

Fig. (2). Polyanionic substances targeted at the viral envelope glycoprotein gp120: PVAS, PAVAS, PRO2000 and JM1590; also targeted at the cellular CD4 receptor: cosalane analogues.
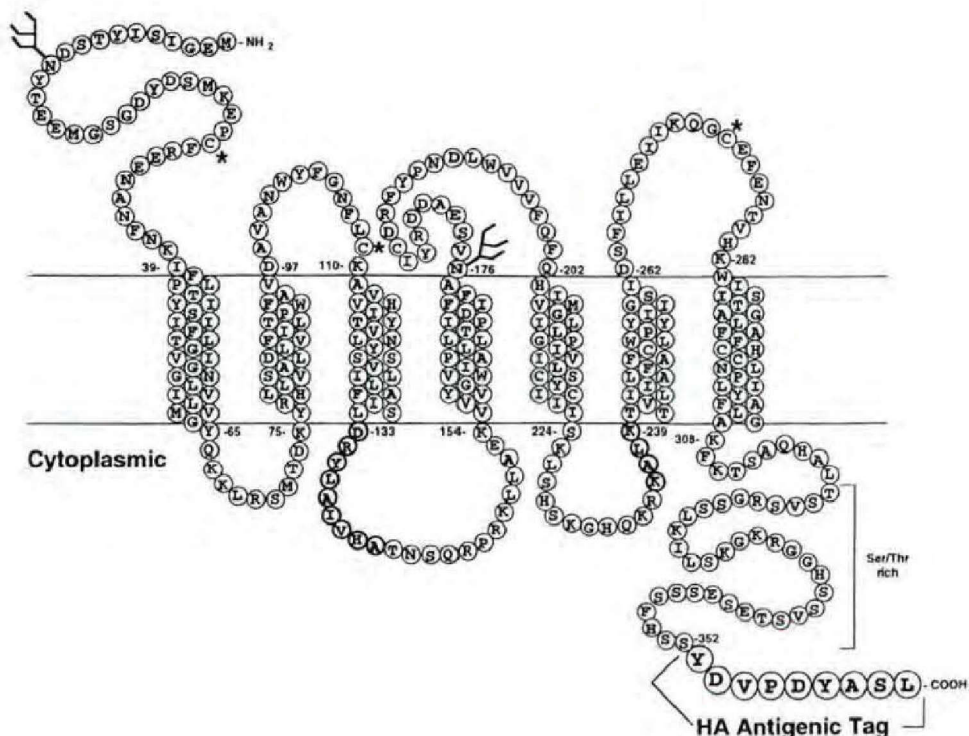
CXCR4: coreceptor for X4 HIV-1 strains
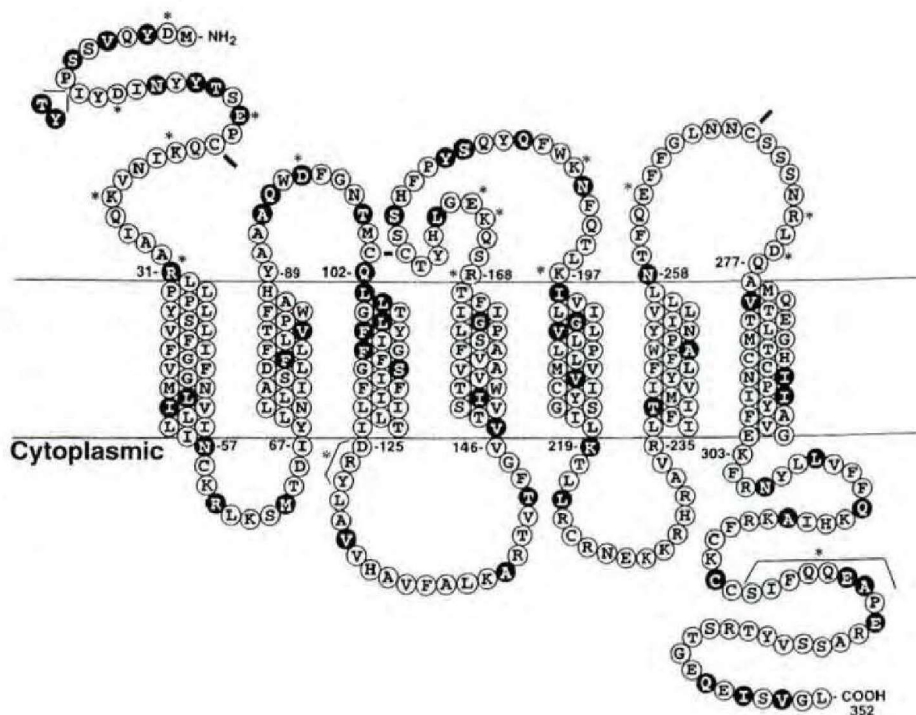
CCR5: coreceptor for R5 HIV-1 strains

Fig. (3). CXCR4: receptor for the CXC chemokine SDF-1, and coreceptor for T-tropic (X4) HIV-1 strains. CCR5: receptor for the CC chemokines MIP-1α, MIP-1β, RANTES, and coreceptor for M-tropic (R5) HIV-1 strains. Figure taken from refs. 40 and 41.

inflammatory proteins"), whereas for CXCR4 only one natural ligand, namely SDF-1 ("stromal-cell derived factor") has been identified. RANTES, MIP-1α and MIP-1β block the entry of M-tropic, whereas SDF-1 blocks the entry of T-tropic HIV strains [42]. Of these chemokines, the LD78β isoform of MIP-1α has emerged as the most potent

chemokine for inhibiting HIV-1 infection [43,44]. LD78β can be considered as a potentially important drug candidate for the treatment of infections with R5 HIV-1 strains.

TAK-779, a quaternary ammonium derivative (Fig. **4**) is the first non-peptidic molecule that has been described to
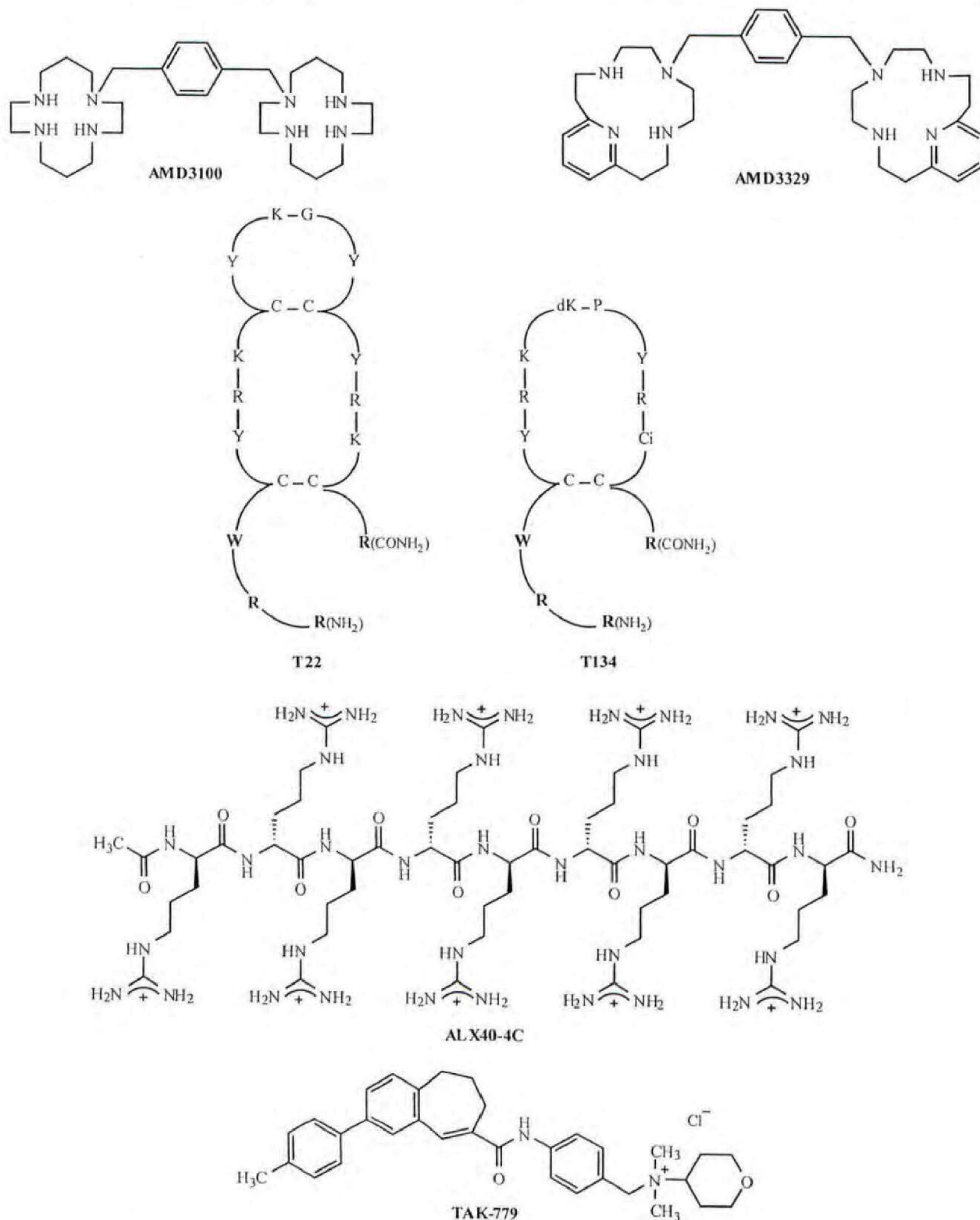

Fig. (4). CXCR4 antagonists: AMD3100, AMD3329, T22, T134 and ALX40-4C. CCR5 antagonist: TAK-779. TAK-779 corresponds to *N,N*-dimethyl-N-[4-[[[2-(4-methylphenyl)-6,7-dihydro-5*H*-benzocyclohepten-8-yl]carbonyl]amino]benzyl] tetrahydro-2*H*-pyran-4-ammonium chloride.

block the replication of M-tropic R5 HIV-1 strains at the CCR5 level [45]. A binding site for this molecule has been identified within the transmembrane helices of CCR5 [46]. TAK-779 has been found to inhibit R5 HIV-1 strains in the nanomolar concentration range, while not affecting X4 HIV-1 strains at 10,000-fold higher concentrations [45]. TAK-779 is not a "pure" CCR5 antagonist, as it also demonstrates some antagonism towards CCR2b. Unlike RANTES, TAK-779 does not induce internalization of CCR5. Its therapeutic potential for HIV-1 infections, remains to be further explored.

Almost simultaneously [47-49], three compounds, i.e. the bicyclam AMD3100 (Fig. **4**) [47], [Tyr-5,12,Lys-7]polyphemusin or T22 (Fig. **4**) [48] and the nonapeptide [49] (D-Arg)$_9$ or ALX40-4C were announced as CXCR4 antagonists, blocking the replication of T-tropic X4, but not M-tropic R5, HIV-1 strains through selective antagonism of CXCR4. Yet, the third compound (ALX40-4C) cannot be regarded as truly specific for CXCR4, since through its highly cationic character, it also interferes with the entry of viruses other than HIV. The second compound (T22), an 18-amino-acid peptide, can be shortened to 14 amino acids, as in T134 (Fig. **4**), without loss of activity [50]. It has also been claimed that T134 would be active against AMD3100-resistant HIV-1 strains [51], but this claim has not (yet) been substantiated. Whether T22 and/or T134 offer potential in the prevention and/or therapy of HIV infections is another issue that needs to be further addressed.

The bicyclams are the most specific and most potent CXCR4 antagonists that have been described to date [52,53]. The *p*-phenylenebis(methylene)-linked dimer of the py[iso-14]ane N$_4$ (AMD3329) (Fig. **4**) displayed the highest antiviral activity of the bis-azamacrocyclic analogues reported [53], exhibiting a 50% effective concentration (EC$_{50}$) against HIV-1 and HIV-2 of 0.8 and 1.6 nM, respectively, that is about 3- to 5-fold lower than the EC$_{50}$ of AMD3100.

The bicyclams had been known as potent and selective HIV inhibitors for a number of years [54,55], before their target of action was identified as the CXCR4 coreceptor [47,56,57]. The bicyclam AMD3100 inhibits the replication of X4 HIV-1 strains within the nanomolar concentration range [55]. As it is not toxic to the host cells at concentrations up to 500 μM, its selectivity index, or ratio of 50% cytotoxic concentration (CC$_{50}$) to 50% antivirally effective concentration (EC$_{50}$) can be estimated at > 100,000.

It took more than 60 passages (300 days) in cell culture for the HIV-1 clone NL4-3 to become 300- to 400-fold resistant to AMD3100 [58,59]; the resistant virus had several mutations scattered over the whole gp120 glycoprotein, but primarily clustered in the V3 loop (i.e., R272T, S274R, Q278H, I288V, N293H and A297T). Most, if not all, of these mutations may have contributed to the resistant phenotype, as indicated by recombination experiments with overlapping parts of the envelope gene [60]. It was postulated that the overall, three-dimensional, conformation of gp120, rather than individual amino acid substitutions, is the prime determinant of the resistance/sensitivity profile of HIV strains to bicyclams

[61]. Resistance to AMD3100 (or SDF-1) does not lead to a switch in coreceptor use [62].

A close correlation has been found, over a concentration range of 0.1-1000 ng/ml, between the AMD3100 concentrations required to inhibit (i) HIV-1 NL4-3 replication, (ii) monoclonal antibody (mAb 12G5) binding to the CXCR4 coreceptor, and (iii) SDF-1-induced signal transduction (Ca^{2+} flux), suggesting an intimate relationship between these three parameters [56,57]. The inhibitory effects of AMD3100 on the T-tropic HIV-1 NL4-3 strain have been demonstrated in a wide variety of cells expressing CXCR4, including PBMC, and, *vice versa*, various T-tropic and dual-tropic, but not M-tropic, HIV-1 strains have proven sensitive to AMD3100 in PBMC.

Negatively charged amino acid (i.e. aspartic acid) residues in both the amino-terminus and second extracellular loop (ECL2) of CXCR4 are thought to be involved in the recognition of CXCR4 by X4 HIV-1 strains [63]. Different single amino acid substitutions of a neutral amino acid residue for aspartic acid and of a non-aromatic residue for phenylalanine, in the second extracellular loop (ECL2) or in the adjacent membrane-spanning domain (TM4), were associated with resistance to AMD3100 [64]. From these studies a model emerged for the interaction of AMD3100 with CXCR4, whereby the positive charges of the bicyclams interact with the aspartic acid residues of ECL2 and TM4, whereas the aromatic linker [i.e., phenylenebis(methylene) in AMD3100] might engage in hydrophobic interactions with the Phe-X-Phe motifs in ECL2 and TM4.

Feline immunodeficiency virus (FIV), that predominantly uses CXCR4 for entering its target cells, is, just like HIV, highly sensitive to inhibition by the bicyclams [65]. The high anti-FIV potency and selectivity of the bicyclams may serve as the starting point for establishing an appropriate therapy for the treatment of FIV infections in cats, but, furthermore, it may prove most valuable as a model to delineate novel strategies to prevent AIDS progression in humans. When the bicyclam AMD3100 was added to PBMC infected with clinical HIV isolates displaying the syncytium-inducing (SI) phenotype, these strains reverted to the non-syncytium-inducing (NSI) phenotype, and, concomitantly, these strains switched from CXCR4 to CCR5 coreceptor use [66]. These findings indicate that selective blockade of CXCR4 by AMD3100 may prevent the switch from the less pathogenic M-tropic R5 to the more pathogenic T-tropic X4 strains of HIV, that *in vivo* heralds the progression to AIDS.

AMD3100 has proved efficacious, alone and in combination with other anti-HIV drugs, in achieving a marked reduction in viral load in the SCID-hu Thy/Liv mouse model [67]. Its *in vivo* activity against T-tropic HIV infections has thus been demonstrated. Following a phase I clinical trial for safety in normal healthy volunteers [68], AMD3100 has recently entered phase II clinical trials in HIV-infected individuals. AMD3100 can be considered as a highly specific CXCR4 antagonist, which consistently blocks the outgrowth of all HIV variants (X4 and dual-tropic X4/R5) that use the CXCR4 receptor for entering the cells (lymphocytes or macrophages/monocytes) [69].
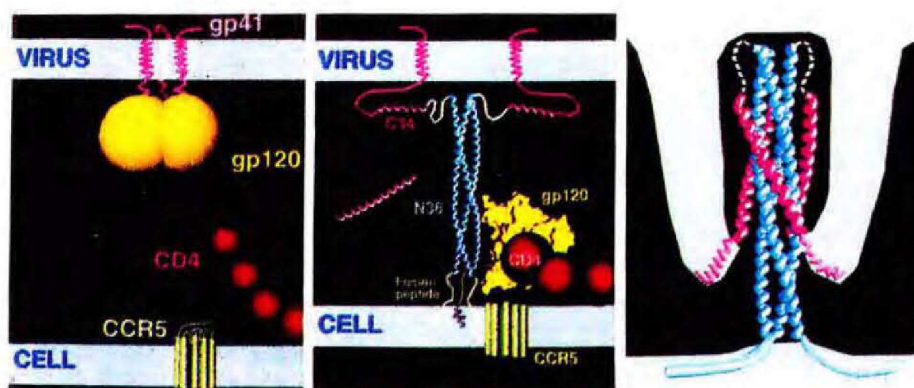
Fig. (5). The HIV-1 entry process: sequential binding of the HIV-1 gp120 glycoprotein to CD4 and then to the coreceptor (CCR5 or CXCR4) is believed to induce conformational changes in the envelope glycoprotein complex (middle panel as compared to left panel). In the hypothetical intermediate (model depicted in the middle panel), the N36 coiled coil (blue) in the gp41 ectodomain is apparent, and the gp41 amino termini ("fusion peptides") are interacting with the target cell membrane. The hydrophobic grooves on the outer face of the N36 coiled coil are unoccupied and available for binding with either the gp41 C34 helices (magenta) or extraneous inhibitors, one of which (magenta) is depicted on the left side in the middle panel. The panel on the right depicts the association of the C34 helices (magenta) and the N36 coil (blue) resulting in the approximation of the viral and target cell membranes. Figure taken from ref. 70.

VIRAL FUSION (gp41) INHIBITORS

The interaction of the X4 or R5 HIV-1 envelope glycoprotein gp120 with the coreceptor CXCR4 or CCR5, respectively, is followed by a spring-loaded action of the viral glycoprotein gp41 (normally covered by the bulkier gp120), that then anchors through its amino termini (the "fusion peptides") into the target cell membrane. This initiates the fusion of the two lipid bilayers, that of the viral envelope with that of the cellular plasma membrane (Fig. 5) [70]. At the onset of the fusion process, the hydrophobic grooves on the surface of the N36 coiled coil and the surface of the N36 pocket, in the gp41 ectodomain become available for binding with either the gp41 C34 helix, or extraneous inhibitors, such as D-amino acid (16- to 18-residue) peptides (i.e., "D10-PX-2K") that bind to the N36 pocket [71] or DP-178 (T-20), a 36-residue peptide that binds to the hydrophobic groove of N36 [70].

T-20 (pentafuside) is a synthetic, 36-amino acid peptide corresponding to residues 127-162 of the ectodomain of gp41 (or residues 643-678 in the gp160 precursor) (Fig. 6). T-20, previously called DP-178, was modeled after a specific domain (within gp41) predictive of α-helical secondary structure: DP-178 consistently afforded 100% blockade of virus-mediated cell-cell fusion (syncytium formation) at concentrations ranging from 1 to 10 ng/ml, i.e., 10^4- to 10^5-fold lower than the cytotoxic concentration [72,73]. In an effort to understand the mechanism of action of DP-178, resistant variants of HIV-1 were generated by serial passage of the virus in the presence of increasing doses of the peptide. These resistant variants revealed mutations in a contiguous 3-amino acid stretch (GIV $\rightarrow$ SIM, DIM, DTV) at positions 36-38 within the amino terminal heptad motif of gp41 [74].

Although the current hypothesis [75] is that T-20 inhibits virus-cell fusion by preventing the formation of the stable coiled coil intermediate (Fig. 5), determinants of coreceptor specificity within the gp120 V3 loop can modulate T-20 efficacy. In particular, the interaction of the gp120 V3 loop with CXCR4 may contribute to the sensitivity to T-20, as X4 HIV-1 strains were found to be, at an average 0.8 $\log_{10}$-fold more sensitive to T-20 than R5 viruses [76]. In addition, it has been suggested that T-20 may interfere with membrane fusion at a post lipid-mixing stage [75].

An initial clinical trial has been carried out with T-20 at four doses (3, 10, 30 and 100 mg twice daily, intravenously, for 14 days) in sixteen HIV-infected adults: at the highest dose (100 mg, twice daily), T-20 achieved by the 15th day a 1.5- to 2.0-fold reduction in plasma HIV RNA [77]. These data provide proof-of-concept that HIV fusion inhibitors are able to reduce virus replication *in vivo*. In trials of 28 days or less T-20 has been given as twice-daily subcutaneous injections or continuous infusion, and in one trial, with a single patient, the compound has been given for more than 28 days in combination with other anti-HIV agents [78]. In the latter study, 1000-fold suppression of HIV-1 RNA was maintained for 20 weeks, no evidence of genotypic resistance to T-20 was observed, and no anti-T-20 antibodies were detected after 28 weeks of administration of T-20 [78]. Meanwhile, T-20 has proceeded to phase II clinical trials, and phase I clinical trials have been initiated with T-1249, 39 mer peptide derived from DP-107, a 38 amino acid peptide corresponding to residues 558-595 of gp160; T-1249 would be 10-fold more potent than T-20 when evaluated (*in vitro*) under the same circumstances [79].

Another polypeptide that may interact directly with gp41 is siamycin (Fig. 6). Siamycin is a tricyclic 21-amino acid peptide isolated from *Streptomyces*: three varieties of siamycin have been described [siamycin II (RP 71955, BMY-29303) [80-82], NP-06 (FR 901724) [83,84] and
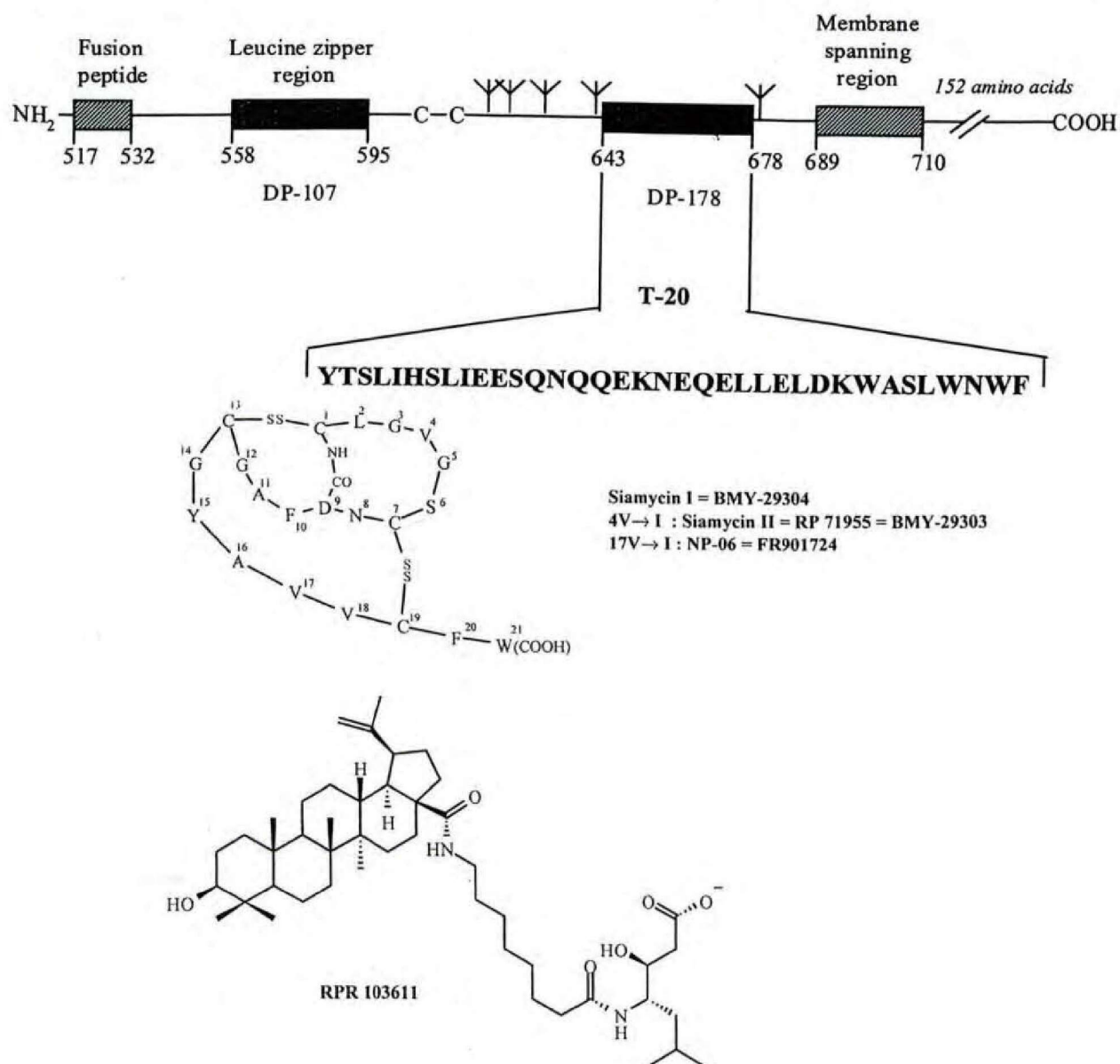
YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF

Siamycin I = BMY-29304
4V → I : Siamycin II = RP 71955 = BMY-29303
17V → I : NP-06 = FR901724

RPR 103611

Fig. (6). Compounds targeted at the transmembrane (TM) glycoprotein gp41: T-20, Siamycins and RPR 103611.

siamycin I (BMY-29304) [82,85]], which differ from one another only at position 4 (V or I) or position 17 (again, V or I). The siamycins have been found to inhibit HIV infection *in vitro* and qualify as fusion inhibitors also, because they exert a strong inhibitory effect on syncytium formation while only weakly interfering with virus-cell binding [83,84]. Siamycin-resistance mutations have been detected in gp41, thus pointing to gp41 as a likely target for the action of siamycin II [85]. There is, in fact, some homology between siamycin II and residues 608-628 of gp41 (amino acid numbering for the gp160 precursor) [81]. Siamycins may hamper the fusogenic activity of gp41 by different mechanisms: for example, through direct binding to the ectodomain of gp41 and/or induction of conformation changes in this domain. The exact mechanism of action of siamycin remains to be resolved, as do its therapeutic potential and pharmacokinetic profile.

The betulinic acid derivative RPR 103611 (Fig. 6) represents the only non-peptidic low-molecular-weight compound known to block HIV-1 infection through interaction with gp41: this triterpene derivative has been found to inhibit the infectivity of a number of HIV-1 strains in the 10 nM concentration range [86], apparently through interference with a post-binding, envelope-dependent step involved in the fusion of the virus with the cell plasma membrane. The exact mode of action of RPR 103611 remains to be elucidated. Resistance to this compound appears to be associated with the emergence of two amino acid substitutions within gp41 (R → A at position 22, I → S at position 84) [87]. In the context of the HIV-1 strain LAI (subtype B), the I84S mutation would be sufficient for drug resistance [87]. Also, the L91H mutation would impart resistance to RPR 103611 [88]. Both I84 and L91 are located in the "loop region" of gp41 separating the proximal

and distal helix domains. The antiviral efficacy of RPR 103611 therefore depends on the sequence, and accessibility, of the gp41 loop region [88].

RPR 103611 may be regarded as an interesting lead compound in the pursuit of non-peptidic low-molecular-weight HIV fusion inhibitors targeted at gp41. Recently, a stereoisomer of RPR 103611, namely IC 9564 (4S-[8-(28-betuliniyl)amino octanoylamino]-3R-hydroxy-6-methylhepta-noic acid), has been found to inhibit HIV replication through interference with the viral envelope-induced membrane fusion [89]: in this case, the viral gp120 was thought to play a key role in anti-HIV activity, since two mutations (G237R and R252K) in gp120 affected the viral sensitivity to the compound [89].

Using molecular docking techniques, the search has begun for small molecules that are targeted at hydrophobic cavity within the gp41 core [90]. This search has already yielded two compounds (ADS-J1 and ADS-J2) having inhibitory activity at μM concentrations on the formation of the gp41 core structure and on HIV-1 infection [90]. However, ADS-J1 and ADS-J2 can be considered as tetra- and trisulfonates, respectively, and are thus reminiscent of other polysulfonates that have been shown to inhibit virus-cell binding through interference with gp120.

NUCLEOCAPSID PROTEIN (NCp7) Zn FINGER-TARGETED AGENTS

The two zinc fingers [Cys-X_2-Cys-X_4-His-X_4-Cys (CCHC), whereby X = any amino acid] in the nucleocapsid

(NCp7) protein (Fig. **7**) [91] comprise the proposed molecular target for zinc-ejecting compounds (Fig. **8**) such as NOBA (3-nitrosobenzamide) [92,93], DIBA (2,2'-dithiobisbenzamide) [94,95], SRR-SB3 (cyclic 2,2'-dithiobisbenzamide) [96], dithiane (1,2-dithiane-4,5-diol,1,1-dioxide,cis) [97] and ADA (azodicarbonamide) [98,99]. These compounds should be able to interfere with both early (uncoating, disassembly) and late phases (packaging, assembly) of retrovirus replication. Their effect at the late phase (assembly) [100] would result in abnormal processing of the gag precursors, due to the formation of intermolecular cross-links among the zinc fingers of adjacent NCp7 molecules and the release of noninfectious virus particles [95]. Their effect at the early phase (disassembly) may also be ascribed to such cross-linkage among adjacent zinc fingers. The DIBAs are able to enter intact virions, and the cross-linkage of NCp7 in virions correlates with loss of infectivity and decreased proviral DNA synthesis during acute infection [101].

The relative rates of HIV inactivation by various DIBAs correlate with their relative kinetic rates of NCp7 zinc ejection, which is consistent with the nucleocapsid protein being the target of action of these compounds. However, DIBAs can be easily reduced yielding two monomeric (R-SH) structures that no longer react with the zinc fingers. Therefore, non-dissociable tethered dithiane compounds have been conceived [97]: these dithiane compounds, like the DIBAs, specifically attack the retroviral zinc fingers; they directly inactivate HIV-1 virions and block production of infectious virus from chronically infected cells. Similarly, macrocyclic diamides possessing a disulfide linkage, as in
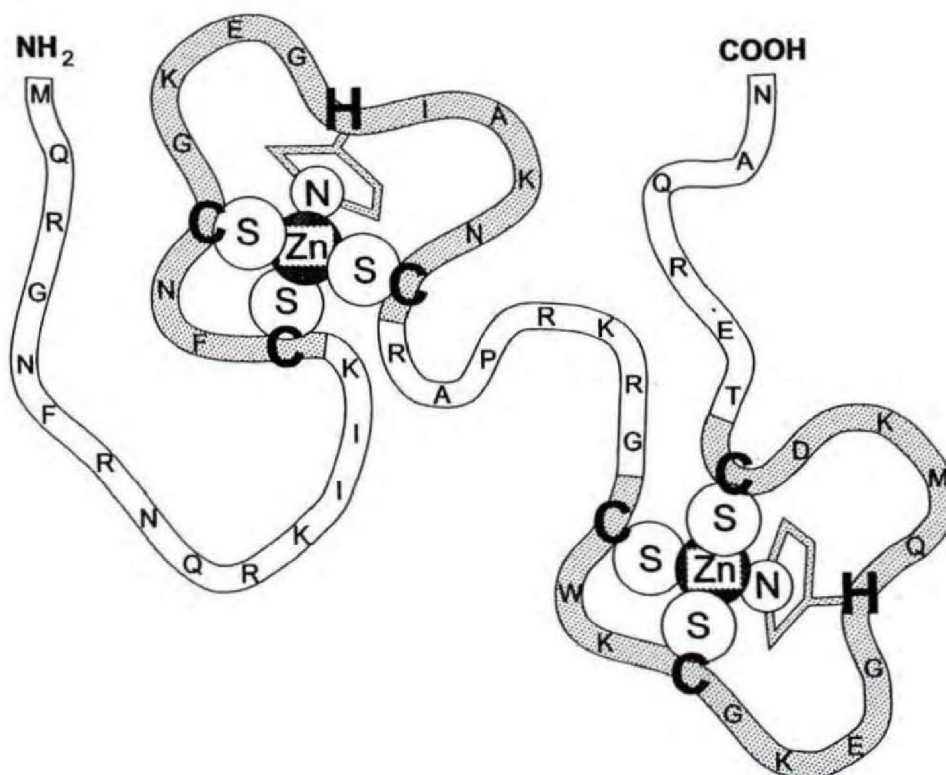

Fig. (7). HIV-1 p7 nucleocapsid (NCp7) protein with the two zinc finger domains. Figure taken form ref. 91.
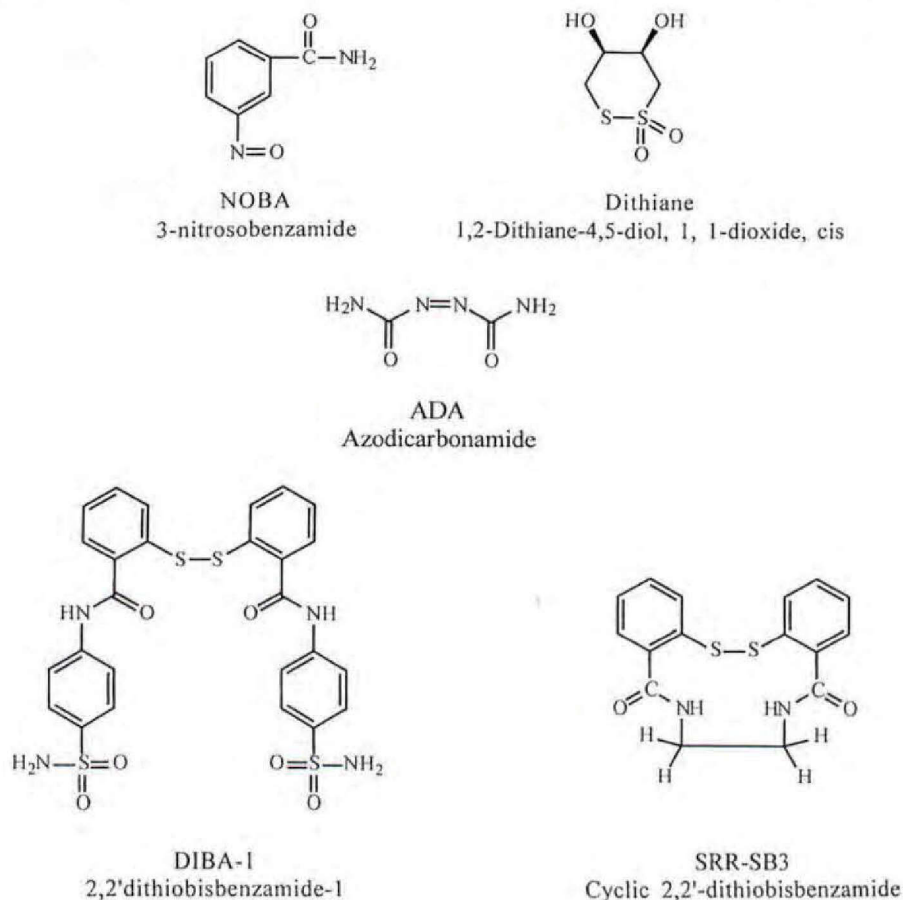
Fig. (8). Compounds targeted at the NCp7 protein Zn fingers: NOBA, Dithiane, ADA, DIBA-1 and SRR-SB3.

SRR-SB3 (Fig. **8**), have been found to inhibit a late stage of the HIV replicative cycle, which most probably corresponds to the viral RNA packaging (assembly) process [96]. Zn-finger-reactive compounds, such as DIBAs and SRR-SB3, may be assumed to interfere with viral infectivity at the level of either virus disassembly or assembly, or both, depending on the accessibility of NCp7 for these compounds during the processes of disassembly and assembly, respectively.

Although NOBA, DIBA, dithiane and ADA have been shown to dock nicely on the NCp7 Zn finger domains [102], and are believed to selectively target these Zn fingers without affecting the cellular Zn finger proteins, their selectivity indexes [ratio of CC_{50} (50% cytotoxic concentration) over EC_{50} (50% effective concentration)] are not that impressive [102]. Of the NCp7-targeted compounds, ADA has been the first to proceed to phase I/II clinical trials in advanced AIDS patients. The results of these trials will soon be revealed. Given the nature of the compound, it will be difficult to pinpoint any *in vivo* antiviral response to ADA to a specific molecular target. ADA may well interact with a variety of targets, and, certainly its inhibitory effects on T-cell responses *in vitro* and *in vivo* [103] can hardly be attributed to an action targeted at the NCp7 Zn fingers.

The nucleocapsid protein NCp7 can also be targeted by compounds that are completely different from the NCp7 zinc ejectors: *viz*, cyclic hexapeptides, such as c(F-C-dW-R-C-K)

that mimic several essential biological determinants (Phe-16, Arg-26, Arg-32 and Trp-37) of NCp7, have been reported to inhibit HIV-1 replication by impairing the formation of a functional complex comprising NCp7, reverse transcriptase, and nucleic acids [104]. While an interesting approach, the affinity, selectivity and bioavailability of the NCp7 peptidomimetics should be considerably improved (by combinatorial chemistry) to provide a drug candidate for clinical investigation.

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

The substrate (dNTP) binding site of the HIV-1 reverse transcriptase (RT) is the target for a large variety of NRTI analogues, which have for several years [105] been recognized as efficacious agents for the treatment of HIV infections: i.e., zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), lamivudine (3TC), abacavir (ABC). Other agents in clinical trial include tenofovir (PMPA) and emtricitabine [(-)FTC]. As a rule, all of these compounds must be phosphorylated to their 5'-triphosphate form, before they can act as competitive inhibitors/substrate analogues/chain terminators at the reverse transcriptase level (Fig. **9**). In contrast to the nucleoside analogues, the nucleotide analogues PMEA and PMPA (Fig. **10**) are already equipped with a phosphonate
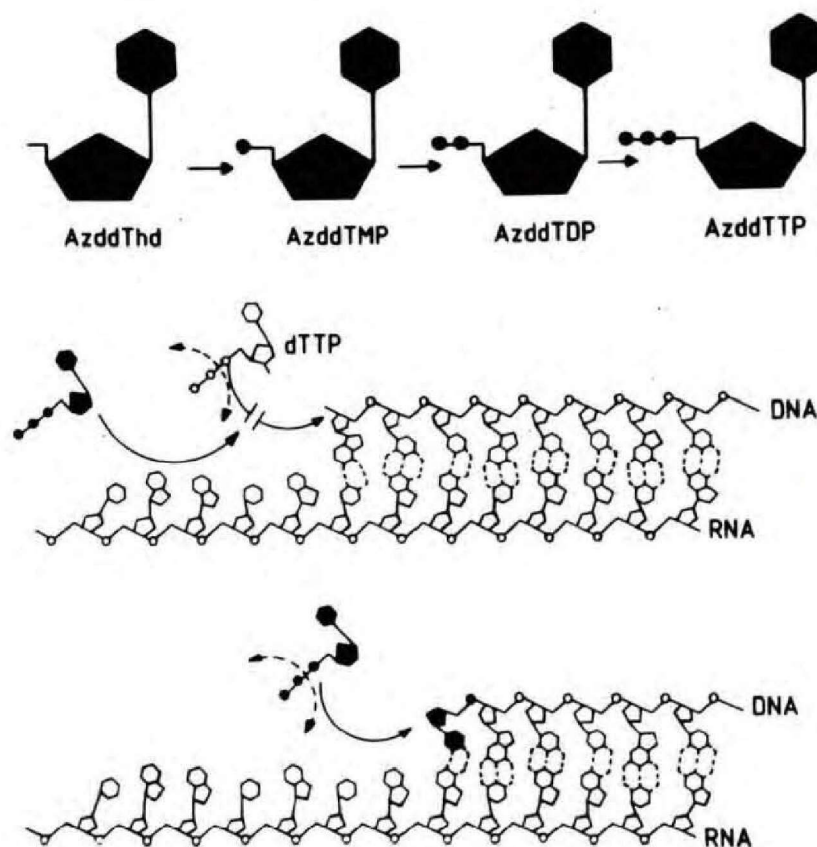
Fig. (9). Mechanism of anti-HIV action of 2',3'-dideoxynucleoside analogues, as exemplified for zidovudine (azidothymidine, AZT, AzddThd).

group, and thus only need two phosphorylation steps to be converted to the active metabolite [106]. From PMEA and PMPA the oral prodrug forms [bis(POM)-PMEA or adefovir dipivoxyl, and bis(POC)-PMPA or tenofovir disoproxil fumarate, respectively] have been prepared. The former is now in phase III clinical trials for the treatment of hepatitis B virus (HBV) infections, whereas the latter has gone through several phase III clinical trials for the treatment of HIV infections [107]. Tenofovir has proven to be antivirally active in heavily antiretroviral-experienced patients; it has a favorable activity/resistance profile in that it by itself leads to little, if any, resistance, while retaining full activity against HIV-1 RT mutations that have developed resistance to 3TC (M184V mutants) or multinucleosids (Q151M mutants) [108].

In addition to 3TC and (-)FTC, the structurally related 2'-deoxy-3'-oxa-4'-thiocytidine (BCH-10652, dOTC) [109], the dioxolane purine nucleoside analogues [110] and the methylenecyclopropane nucleoside analogues (and their phosphoro-L-alaninate diesters) [111,112] have recently been described as new anti-HIV agents. Emtricitabine [(-)FTC] is in phase III trials for HIV and phase I/II trials for HBV; it is considered for use in the multidrug combination therapy of HIV-1 and HBV infections [113]. DAPD, (-)-β-D-2,6-diaminopurine dioxolane, which is converted by adenosine deaminase to (-)-β-D-dioxolane guanine (DXG) (which is then further metabolized intracellularly to its active

metabolite DXG-TP, a strong alternative substrate inhibitor of the HIV RT) [114], has proven active against AZT- and 3TC-resistant HIV-1 strains and has proceeded to phase I/II clinical studies [115]. BCH-10652 (dOTC) has demonstrated activity against HIV-1 in the SCID-hu Thy/Liv model; despite its structural similarity to 3TC it proved also active against 3TC-resistant HIV-1 (M184V), albeit at a relatively high dosage level (400 mg/kg/day) [116].

All the nucleoside analogues mentioned above may ultimately, upon their intracellular metabolism, act as chain terminators, akin to AZT (Fig. 9). Chain-terminating nucleotides inhibit HIV replication because their incorporation at the 3' end of the nascent DNA chain prevents further elongation. However, it has been recently shown that under physiological conditions RT can remove these chain terminators and thus unblock the primer termini [117,118]. Mutant RTs associated with AZT resistance would be capable of removing the chain terminating residue with much greater efficiency than wild-type RT and this should, in turn, facilitate rescue of DNA synthesis. On the other hand, the M184V mutation, which confers high-level resistance to 3TC, would severely impair the removal of chain-terminating nucleotides and hence suppress the rescue of AZT-terminated DNA synthesis [119]. Similarly, foscarnet resistance mutations would also suppress the removal of AZT monophosphate from the blocked
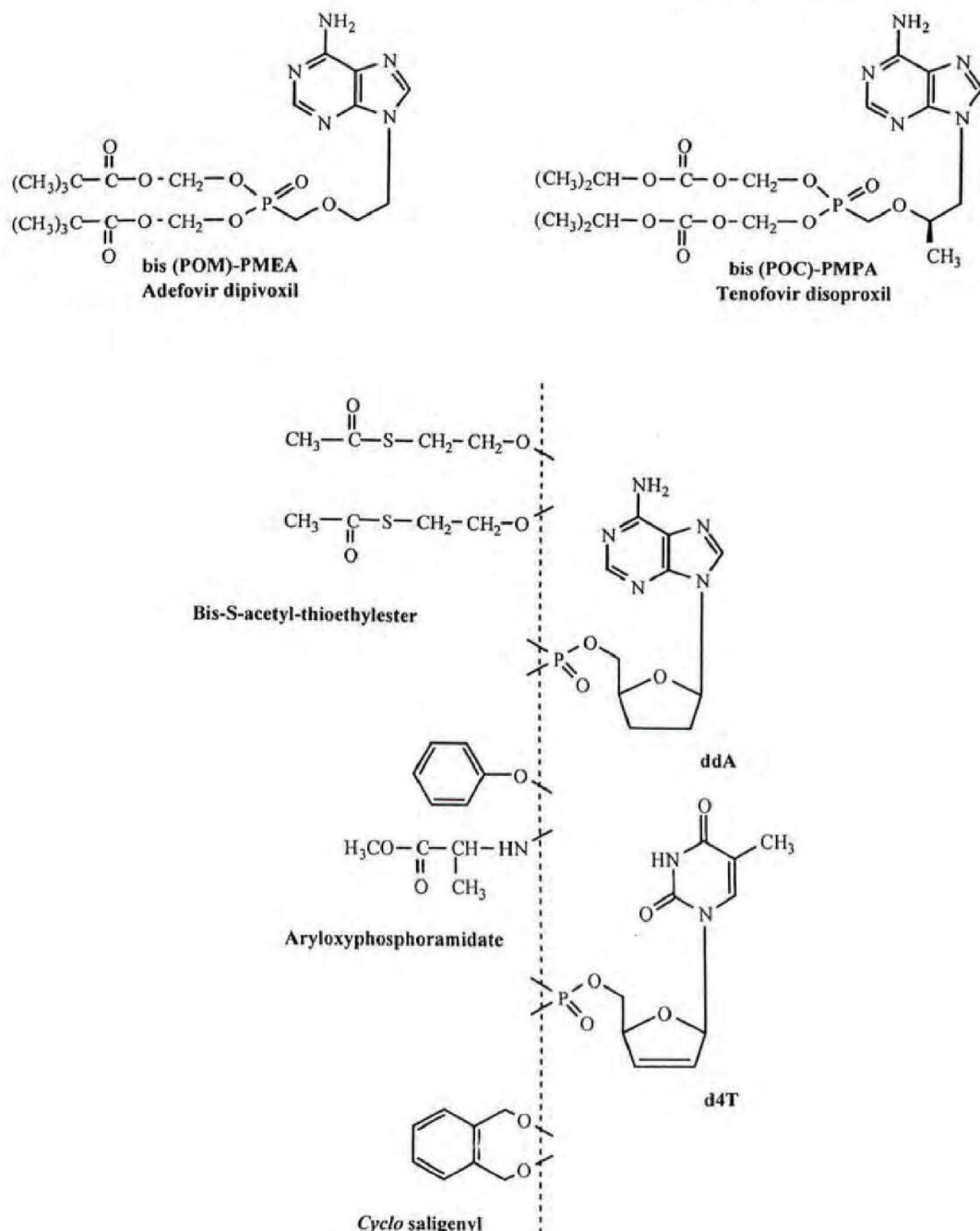
Fig. (10). Acyclic nucleoside phosphonates: PMEA (adefovir) and PMPA (tenofovir) in their prodrug forms, adefovir dipivoxil and tenofovir disoproxil. Prodrugs of ddNMPs: Bis(S-acetyl-thioethyl)ester of ddAMP, (Aryloxy)phosphoramidate of d4T, and *cyclo*Sal-d4TMP and *cyclo*Sal-ddAMP.

primer/templates, thus providing a likely explanation for the resensitization of AZT-resistant HIV-1 strains to AZT [120].

The bottleneck in the metabolic pathway leading from AZT and the other 2',3'-dideoxynucleoside (ddN) analogues to their active 5'-triphosphate form is the first phosphorylation step. Therefore attempts have been made at constructing 2',3'-dideoxynucleotide (ddNMP) prodrugs, that, once they have been taken up by the cells, deliver the nucleotide (ddNMP) form. This approach has proven particularly successful for a number of NRTIs such as 2',3'-dideoxyadenosine (ddA) and d4T (Fig. 10). 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 [121]. By directly delivering bis(SATE)ddAMP into the cells, one circumvents the adenosine deaminase step that would otherwise rapidly degrade ddA to ddI.

Similarly, aryloxyphosphoramidate derivatives (Fig. **10**) of d4T [i.e., So324, a d4T-MP prodrug containing at the phosphate moiety a phenyl group and the methylester of alanine linked to the phosphate group through a phosphoramidate linkage] have been constructed [122-124]. Once taken up by the cells, d4TMP is released intracellularly (*via* the alaninyl d4TMP intermediate) [125].

The latter could be considered as an intracellular depot form of d4TMP [125]. This phosphoramidate prodrug approach does not seem to work so well with zidovudine, where the main metabolite formed from the alaninyl AZTMP intermediate is AZT rather than AZTMP [126] thus explaining why d4T phosphoramidate prodrugs, but not AZT phosphoramidate prodrugs, retain anti-HIV activity in HIV-infected thymidine kinase-deficient cell cultures. In resting monocytes/macrophages (M/M) the aryloxyphosphoramidate derivatives of d4T, d4A and ddA provided an anti-HIV activity that was 25- to 625-fold greater than that of the parent nucleosides (d4T, d4A and ddA) [127].

The thymidine kinase (in the case of d4T) and the adenosine deaminase (in the case of ddA) can also be bypassed by using the cyclic saligenyl approach [128,129]. *Cyclo*Saligenyl pronucleotides of d4T and ddA (Fig. 10) deliver exclusively the nucleotides d4TMP and ddAMP, not only under chemical-simulated hydrolysis conditions but also under intracellular conditions [130,131]. The *cyclo*Sal approach has also been applied, with success, to F-*ara*-ddA (lodenosine, 2'-fluoro-*ara*-2',3'-dideoxyadenosine) [132].

REVERSE TRANSCRIPTASE INHIBITORS TARGETED AT THE ALLOSTERIC NON-SUBSTRATE BINDING SITE

Starting from the HEPT and TIBO derivatives, more than 30 structurally different classes of compounds have been identified as NNRTIs, i.e., compounds that are specifically inhibitory to HIV-1 replication and targeted at a non-substrate binding site of the reverse transcriptase [133]. Three NNRTIs (nevirapine, delavirdine and efavirenz) have so far been formally licensed for clinical use in the treatment of HIV-1 infections, emivirine (MKC-442) is in advanced (phase III) clinical trials, and others are in preclinical or early clinical development. Safety assessment and pharmacokinetics studies have supported the continued development of emivirine as a treatment for HIV-1 infection in both adult and pediatric patient populations [134]. The NNRTIs interact with a specific "pocket" site of the HIV-1 RT (Fig. 11) [135], which is closely associated with, but distinct from, the substrate binding site. The affinity of efavirenz for its binding site has been shown to increase in the following order: free enzyme < (i.e., bound with lower affinity) binary RT-template-primer (TP) complex < ternary RT-TP-deoxynucleoside triphosphate (dNTP) complex [136].

NNRTIs are notorious for rapidly eliciting resistance, resulting from mutations at the amino acid residues that surround the NNRTI-binding site of HIV-1 RT (i.e., L100I, K101E, K103N, V106A, V179D, Y181C, Y188H, G190A, P225H, F227L and P236L. The most common mutations occurring in the clinical setting, i.e. in patients treated with delavirdine (alone) [137] or nevirapine (plus protease inhibitors) [138] are K103N and Y181C. Emergence of NNRTI-resistant HIV strains can be prevented if the NNRTIs are combined with NRTIs and used from the beginning at sufficiently high concentrations [133].

Recent studies with NNRTIs have revealed deeper insights in the structural features required for anti-HIV activity [139-141], and the conformational changes that may help to minimize drug resistance [142], and some new classes of highly potent anti-HIV-1 compounds have been discovered as well. The thiocarboxanilide UC-781 is an exceptionally potent inhibitor of HIV-1 replication [133]. It has been found to restore the antiviral activity of AZT against AZT-resistant HIV-1 [143]. With the advent of the more potent NNRTIs, it has also been demonstrated that NNRTIs are not exclusively specific for HIV-1, but also have limited activity against HIV-2 [144]. If this observation applied also to the most potent NNRTIs (that are active against HIV-1 at nanomolar concentrations), it would make them therapeutically useful against HIV-2 infections.

To the new classes of NNRTIs that offer potent anti-HIV-1 activity belong the thieno[3,4-*e*][1,2,4]thiadiazine derivative QM96521 [145,146], the quinoxaline GW420867X [147], the imidazole derivative S-1153 (AG1549, capravirine) [148-150], (-)-6-chloro-2-[(1-furo[2,3-c]pyridin-5-yl-ethyl)thio]-4-pyrimidinamine (PNU-142721) [151], N-[2-(2,5-dimethoxyphenylethyl]-N'-[2-(5-bromopy-ridyl]-thiourea (HI-236) [152,153], the pyrido[1,2a]indole derivative BCH-1 [154], the 4-cyclopropylalkynyl-4-trifluoromethyl-3,4-dihydro-2(1*H*)qui-nazolinones DPC 961 and DPC 963, the 4-cyclopropylalkenyl-4-trifluoromethyl-3,4-dihydro-2(1*H*)quinazolinones DPC 082 and DPC 083 [155], the thiophene-ethylthiourea (TET) derivative HI-443
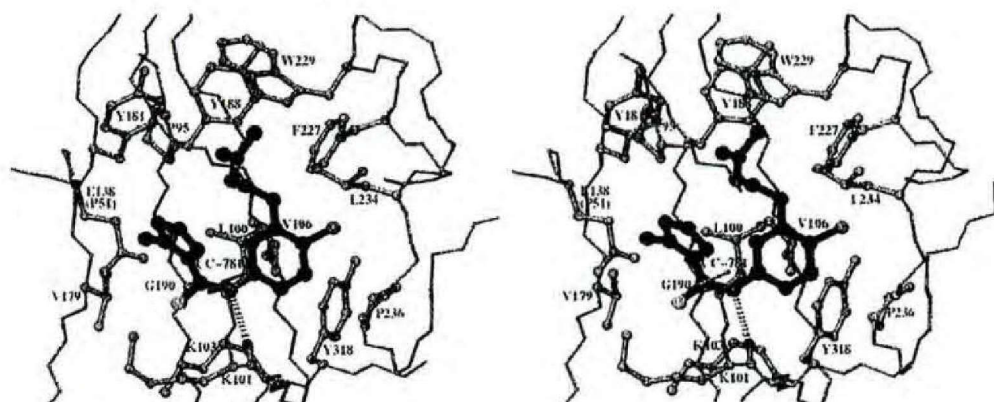

Fig. (11). Stereodiagram of thiocarboxanilide UC-781 positioned in the NNRTI pocket. Figure taken from ref. 135.
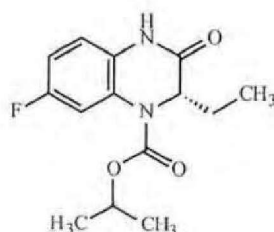
[156], the cyclohexenylethylthiourea derivatives HI-346 and HI-445 [157], the *cis*-cyclopropyl urea-PETT derivatives [158], the alkenyldiarylmethane (ADAM) series of compounds [159], the pyrrolobenzoxazepinone (PBO) derivatives [160], the emivirine (MKC-442) derivatives SJ-3366 [161], GCA-186 [162] and TNK-6123 [162] and the 4"-deaminated TSAO-T (4"-H TSAO-T) [163] (Fig. **12**). Also, novel 2,2-dioxide-4,4-disubstituted-1,3-*H*-2,1,3-benzothiadiazines [164], N-[2-(4-methylphenyl)ethyl]-N'-[2-(5-bromopyridyl]thiourea [165], quinoxalinylethylpyridyl thio-ureas [166], and [AZT]-[TSAO-T] and [d4T]-[TSAO-

T]heterodimers [167] have been described as new NNRTI derivatives.

As a rule, the "new" (or second generation) NNRTIs exhibit higher potency than the "old" (or first generation) NNRTIs against wild-type and NNRTI-resistant HIV-1 strains (carrying the NNRTI resistance mutations K103N, V106A, Y181C, P236L, ...) [149,151,155-157,160,162]. In particular, capravirine is active against the single K103N RT mutation that renders HIV-1 resistant to each of the currently marketed NNRTIs [150]. Other remarkable features include
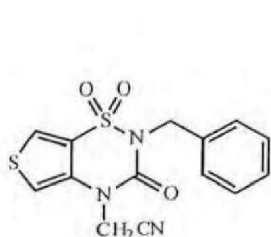
Thienothiadiazine (TTD)
QM96521

Quinoxaline
GW420867X
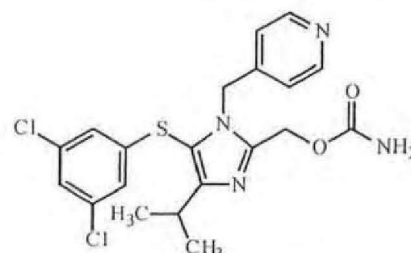
S-1153
AG1549
Capravirine
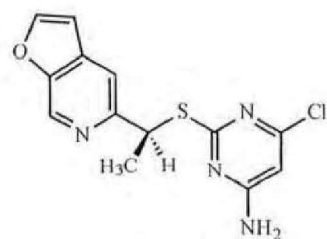
PNU-142721
(-)-(S)-enantiomerderivative
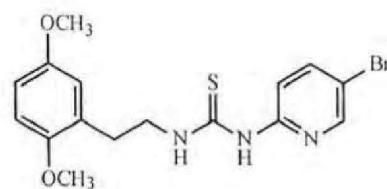
Phenylethylthiourea (PET)
HI-236

Pyrido[1,2a]indole
BCH-1

DPC 961   X= 6-Cl
DPC 963   X = 5,6-diF

DPC 083   X= 6-Cl
DPC 082   X = 5,6-diF

Thiophene-ethylthiourea (TET)
derivative HI-443

Cyclohexenylethylthiourea derivatives
HI-346 (X=Br)
HI-445 (X-Cl)

Cis-cyclopropylurea-PETT derivative

Alkenyldiarylmethane
(ADAM) derivative

(Fig. 12). contd.....

Pyrrolobenzoxazepinone
(PBO) derivative

SJ-3366
Emivirine (MKC-442) derivative

GCA-186
Emivirine (MKC-442) analogue

TNK-6123
Emivirine (MKC-442) analogue

4"-H TSAO-T

BBNH

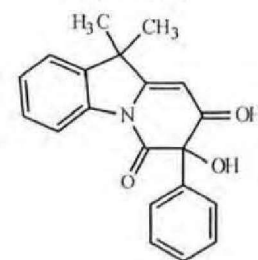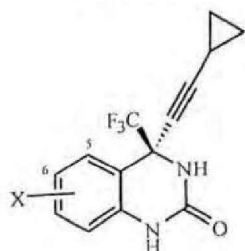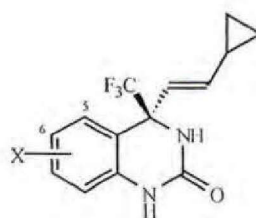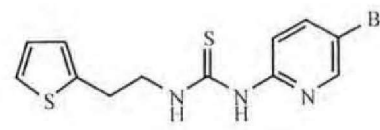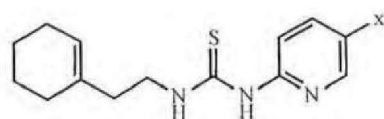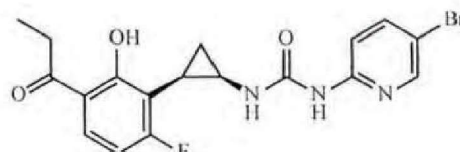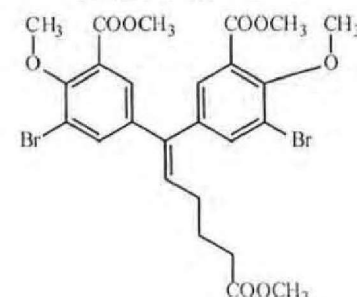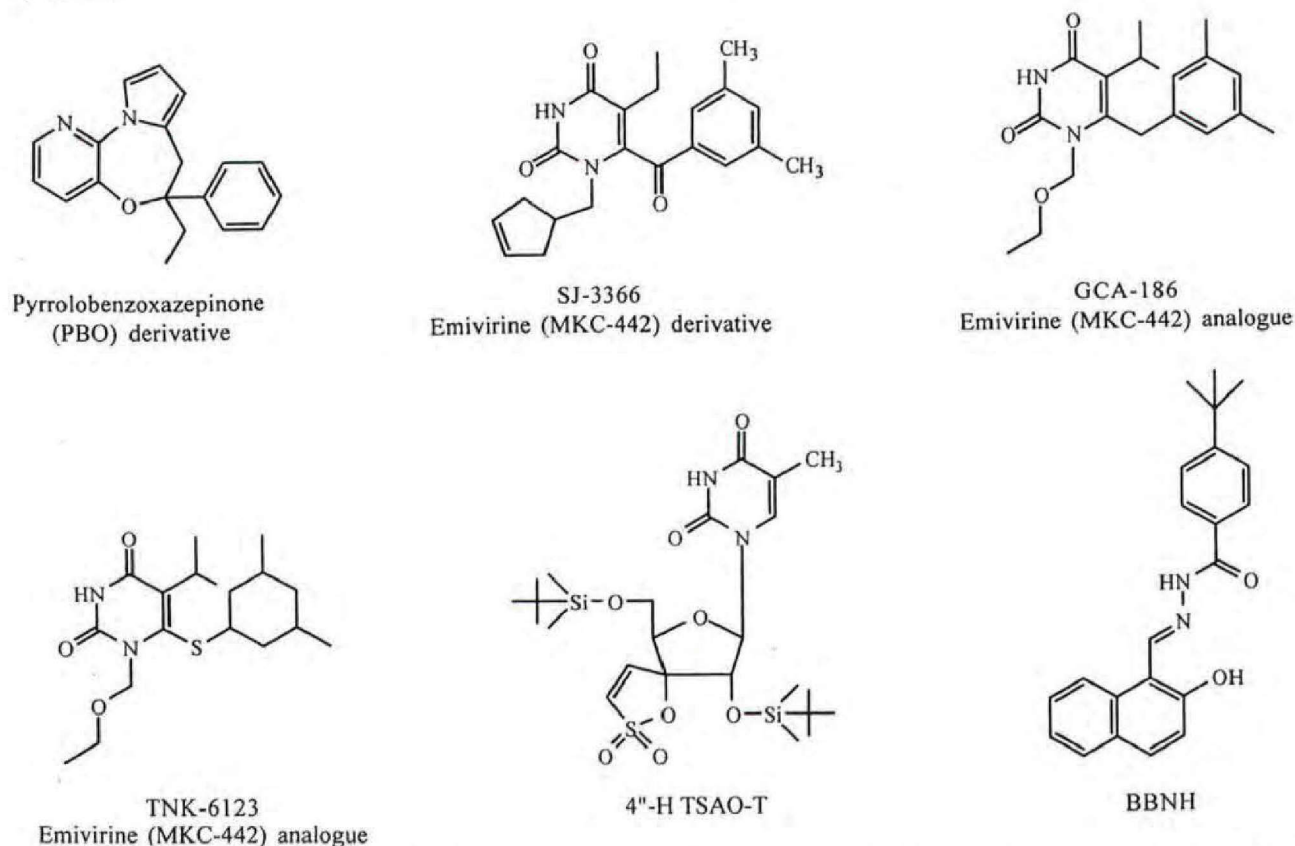**Fig. (12).** Novel NNRTIs (non-nucleoside reverse transcriptase inhibitors): thieno[3,4-*e*][1,2,4]thiadiazine (TTD) QM 96521; quinoxaline GW420867X; capravirine (S-1153, AG1549); PNU-142721 (-)-(*S*)-enantiomer; phenylethylthiourea (PET) derivative HI-236; pyrido[1,2a]indole BCH-1; 4-cyclopropylalkynyl- and 4-cyclopropylalkenyl-4-trifluoromethyl-3,4-dihydro-2(1*H*)quinazolinones DPC 961, 963, 082 and 083; thiophene-ethylthiourea (TET) derivative HI-443; cyclohexenylethylthiourea derivatives HI-346 and HI-445; *cis*-cyclopropylurea-PETT derivative; alkenyldiaryl-methane (ADAM) derivative; pyrrolobenzoxazepinone (PBO) derivative; emivirine (MKC-442) derivatives SI-3366, GCA-186 and TNK-6123; and 4"-deaminated TSAO-T. Ribonuclease H inhibitor: (4-*t*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH).

the exquisite potency of some of the new NNRTIs such as SJ-3366 [161], which was reported to inhibit HIV-1 replication at concentrations below 1 nM with a therapeutic index greater than 4,000,000, and the fact that the NNRTIs cis-cyclopropylurea-PETT [158] and pyrrolobenzoxaze-pinone (PBO) derivatives [160] are orally bioavailable and penetrate well into the brain.

The broad, potent antiviral activity, and favorable pharmacokinetic profile, have led to the selection of PNU-142721 for clinical studies [151]; and DPC 961, DPC 963, DPC 082 and DPC 083 for clinical development [155]. With AG1549 (capravirine) preliminary evidence of efficacy has already been obtained in a phase I study [149]; the compound is now in phase II/III clinical trials [150].

While numerous inhibitors (both NRTIs and NNRTIs) of the DNA polymerase activity of HIV-1 reverse transcriptase have been identified, very little is known about inhibitors of the RT-associated ribonuclease H (RNase H). An example is (4-*t*-butylbenzoyl)-2-hydroxy-1-naphthaldehyde hydrazone (BBNH) (Fig. 12) that inhibits RNase H, and, through interaction with the NNRTI binding site, also the HIV-1 RT DNA polymerase activity [168].

Further development of BBNH has resulted in SP1093V, a water-soluble formulation of the Fe(III) chelate of BBNH incorporated into a block copolymer. SP1093V retains full activity against a variety of NRTI- and NNRTI-resistant HIV-1 isolates, including viral strains with multiple mutations in the NNRTI binding pocket that are resistant to nevirapine, delavirdine and efavirenz. It has so far proved impossible to obtain resistance to SP1093V despite numerous passages of the virus in the presence of the compound [168].

HIV INTEGRASE INHIBITORS

Retrovirus integration requires at least two viral components, the retroviral enzyme integrase, and *cis*-acting sequences at the retroviral DNA termini U3 and U5 ends of the long terminal repeats (LTRs) (Fig. **13**) [169]. Since HIV, like other retroviruses, cannot replicate without integration into a host chromosome, integrase has been considered as an attractive therapeutic target. Numerous compounds have been described as inhibitors of HIV-1 integrase (for a review, see Pommier *et al.*) [169]: for
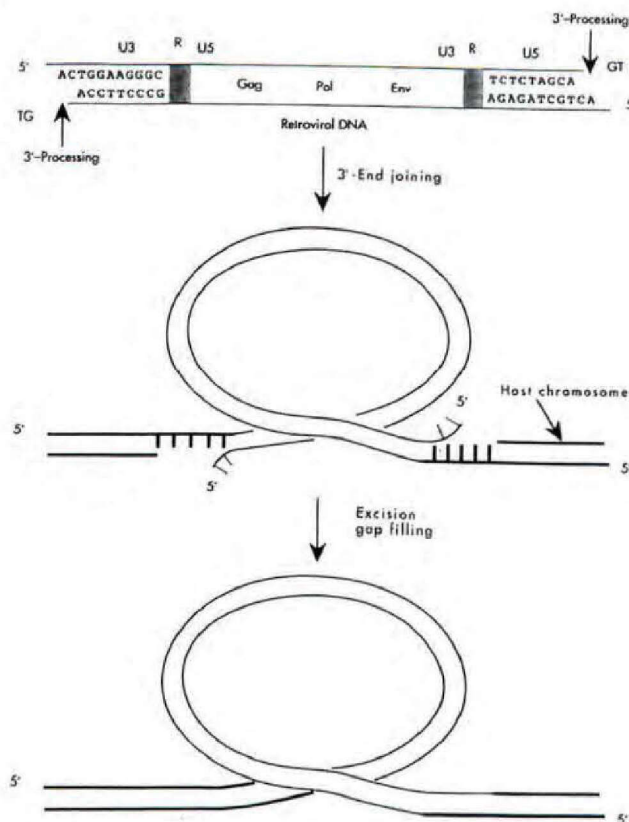
Fig. (13). Retroviral DNA integration: integrase catalyses 3' processing and 3'-end joining (strand transfer). Figure taken from ref. 169.

example, (-)arctigenin (a lignanolide) [170], 2-mercaptobenzenesulfonamides [171], quinalizarin, purpurin, tetracyclines and various fourpoint pharmacophores [172,173], polyamides, bisdistamycins and lexitropsins [174], hydroxybenzoic and hydroxycinnamic acid flavon-3-yl esters [175] and all kinds of cinnamoyl compounds [176], and a number of polyhydroxylated aromatic type of compounds, including ellagic acid, purpurogallin, 4,8,12-trioxatricornan and hypericin [177], and a series of thiazolothiazepine derivatives, preferably possessing the pentatomic moiety SC(O)CNC(O) with two carbonyl groups [178]. The problem with integrase inhibitors is that, while they might be effective in an enzyme-based assay, their anti-HIV activity in cell culture may be masked by cytotoxicity, and if they do exhibit anti-HIV activity, this may be attributed to antiviral actions targeted at other steps in the HIV replicative cycle.

Two pertinent examples of integrase inhibitors (Fig. **14**) that have been amply documented for their capacity to inhibit HIV integrase are zintevir [29-31] and L-chicoric acid [179-181]. As already discussed earlier, zintevir appears to primarily owe its anti-HIV activity in cell culture to an interaction with the viral envelope glycoprotein gp120 [32,33], and this seems also to be the case for L-chicoric acid. L-chicoric acid is structurally reminiscent of curcumin [182], 3,5-dicaffeoylquinic acid [183], rosmarinic acid [184] and dicaffeoyltartaric acids (DCTAs) [185], and all these compounds have been reported to inhibit HIV-1 integrase: i.e., the dicaffeoylquinic acids (DCQAs) do so at

concentrations between 150 and 840 nM, while they inhibit HIV replication at concentrations between 2 and 12 µM [185]. Integrase was identified as the molecular target for the action of L-chicoric acid when a single amino acid substitution (G140S) in the integrase rendered the corresponding HIV-1 mutant resistant to L-chicoric acid [181].

We have recently demonstrated [186] that L-chicoric acid owes its anti-HIV activity in cell-culture to an interaction with the viral envelope gp120. Upon repeated passages of the virus in the presence of the compound, mutations were found in the V2, V3 and V4 loop of gp120, while no mutations were seen in the integrase. We did confirm that in an enzymatic assay L-chicoric acid inhibited HIV integrase activity, but integrase carrying the G140S mutation appeared to be as sensitive to the inhibitory effect of L-chicoric acid as was the wild-type integrase. Furthermore, L-chicoric acid proved inactive against HIV strains that were resistant to polyanionic compounds known to interact at the virus adsorption level, and time-of-addition experiments further corroborated an interaction of L-chicoric acid at the virus adsorption, rather than the proviral DNA integration, stage [186].

Recently, a number of diketo acids (such as L-731,988 and L-708,906) (Fig. **14**) have been described as inhibitors of the integrase-mediated strand transfer reaction that leads to the covalent linkage of the viral DNA 3' ends to the cellular (target) DNA [187]. These compounds were also found to

T G   T G

G—G-G—G

G—G-G—G

T   T G   T

G   3'

5'

L-chicoric acid
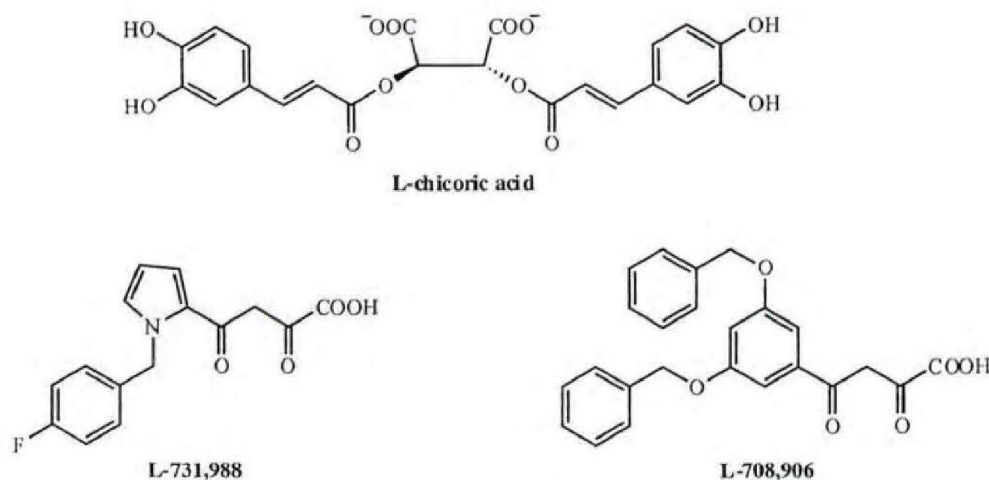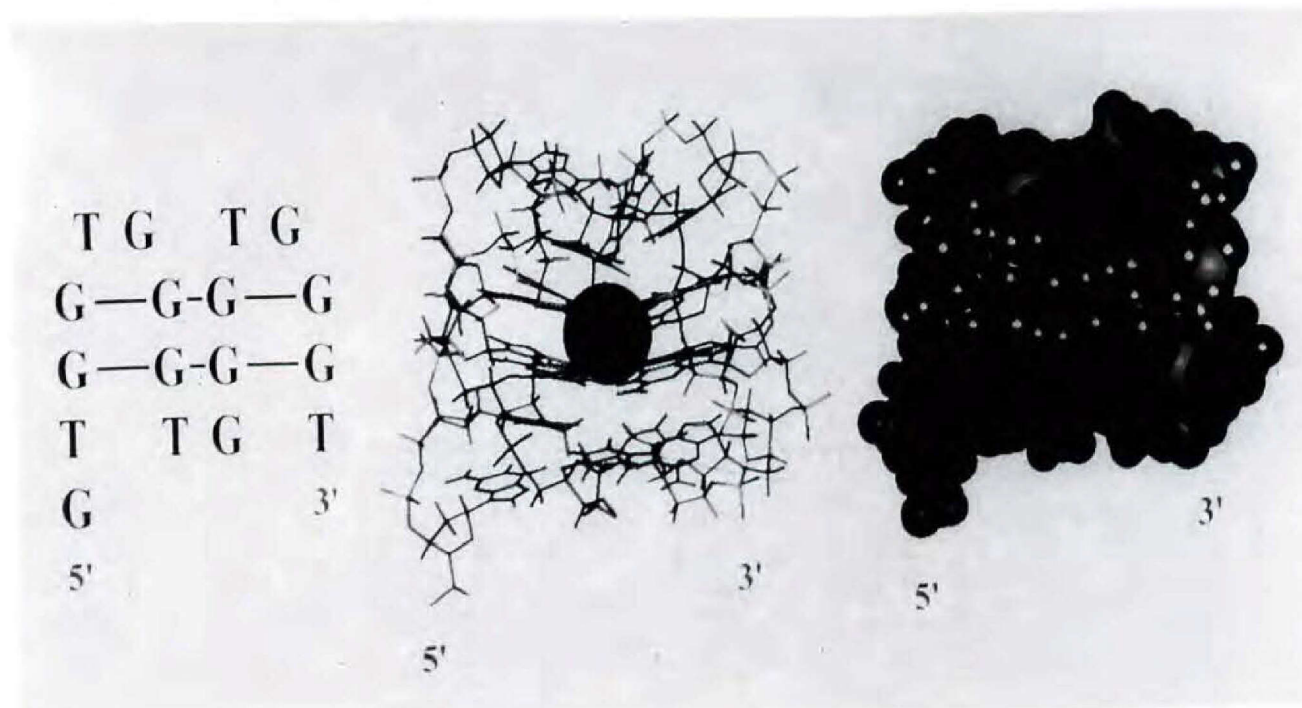
L-731,988

L-708,906

Fig. (14). Integrase inhibitors: Zintevir (AR177, T30177), L-chicoric acid, and the diketo acids L-731,988 and L-708,906.

inhibit HIV-1 replication in cell culture. Furthermore, mutations in the HIV-1 integrase conferred resistance to the inhibitory effects of the compounds on both strand transfer and HIV-1 infectivity [187]. Thus it was surmised that these diketo acids owe their antiviral activity exclusively to inhibition of one of the two catalytic functions of integrase, namely strand transfer [187] (the other catalytic function being endonucleolytic processing of the (pro)viral DNA to remove the terminal dinucleotide (GT) from each 3'end).

TRANSCRIPTION (TRANSACTIVATION) INHIBITORS

At the transcription level, HIV gene expression may be inhibited by compounds that interact with cellular factors that bind to the LTR promoter and that are needed for basal

level transcription, such as the NF-κB inhibitors [188]. Greater specificity, however, can be expected from those compounds that specifically inhibit the transactivation of the HIV LTR promotor by the viral Tat (*trans*-activating) protein [188] (Fig. 15). Tat has pleiotropic effects: it not only activates the transcription of HIV-1 RNA, but also binds to a number of receptors, i.e. on smooth muscle and skeletal muscle cells [189]: the basic domain of Tat (Fig. 15) may be important, not only for translocation but also for nuclear localisation and *trans*-activation, and thus targeting of the Tat basic domain may provide great scope for therapeutic intervention in HIV-1 infection [189].

Given the highly cationic character (i.e. 8 positive charges) of the Tat basic domain, it is not surprising that sulfated polysaccharides would bind avidly to this domain [190]. To the extent that Tat can be released and enter cells

1-	E	P	V	D	P	R	L	E	P	W	K	H	P	G	S	Q	P	K	T	A	(Proline-rich domain)
21-	C	T	T	C	Y	C	K	K	C	C	F	H	C	Q	V	C	F	T	T	K	(Cysteine-rich domain)
41-	A	L	G	I	S	Y	G	R	K	K	R	R	Q	R	R	R	P	P	Q	G	(Basic domain)
61-	S	Q	T	H	Q	V	S	L	S	K	Q	P	T	S	Q	P	R	G	D	P	(RGD C-terminal domain)
81-	T	G	P	K	E																
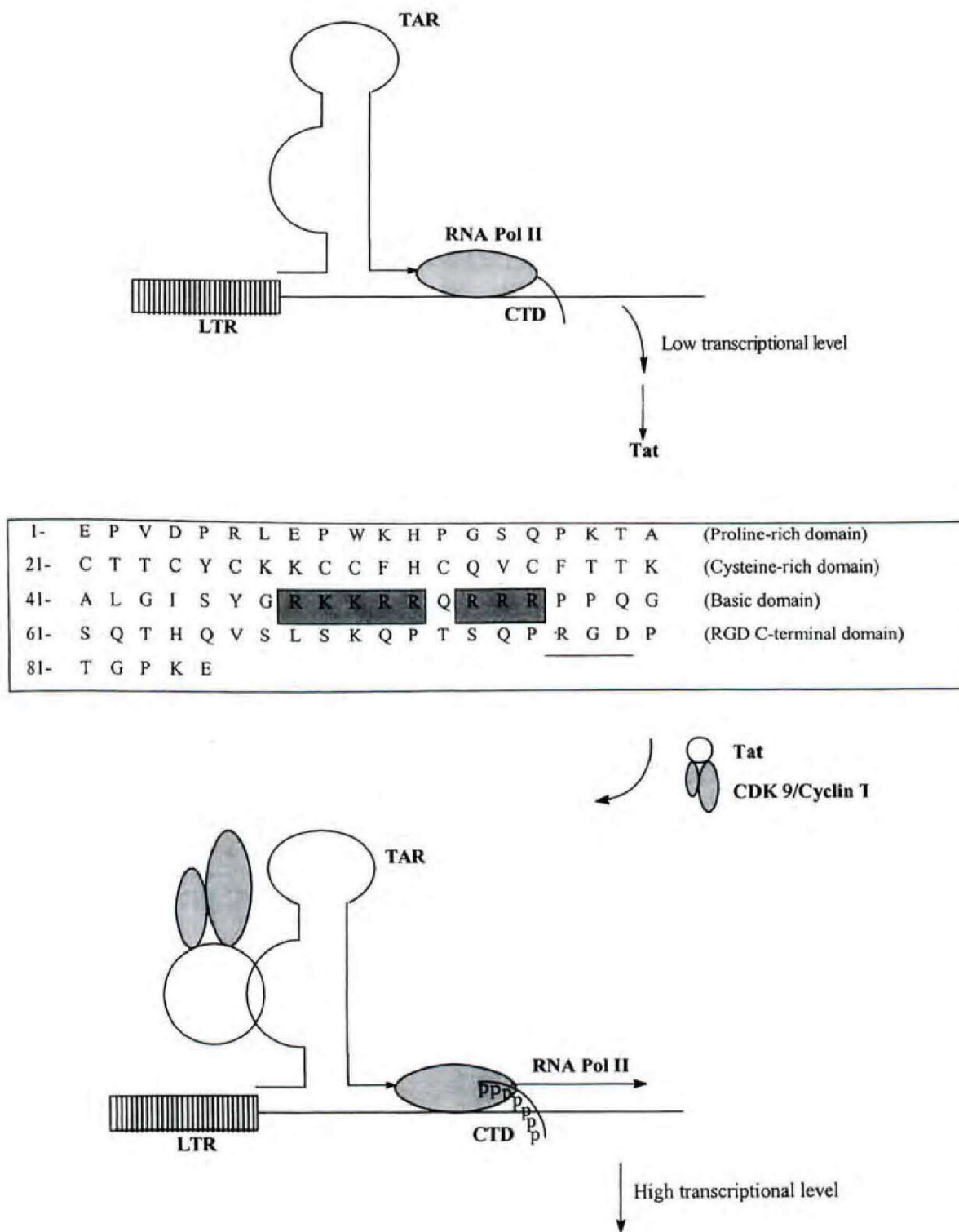
Fig. (15). HIV-1 transcription transactivation process: Tat, the HIV-1 *trans*-activating protein, contains four major domains: proline-rich, cysteine-rich, basic and RGD-containing C-terminal domain. Tat, through its interaction with the TAR RNA element, functions as a potent amplifier of viral gene expression. In the absence of Tat, the transcription level is low because of the hypophosphorylation of the C terminal domain (CTD) of RNA polymerase II. Poorly processive RNA polymerase II is able to synthesize the bulged stem loop structure TAR, which corresponds to the first 80 nucleotides of the transcript. Tat, in a complex with CDK9/cyclin T, binds to TAR. CDK9 then hyperphosphorylates CTD, thus leading to high transcriptional level.

freely, compounds that sequester Tat may offer a therapeutic opportunity, i.e. in suppressing the angiogenic activity of Tat and blocking its stimulating effect on the growth of endothelial tumor cells including Kaposi's sarcoma-derived endothelial spindle cells [190].

A number of compounds have been reported to inhibit HIV-1 replication in both acutely and chronically infected cells through interference with the transcription process: i.e. fluoroquinoline [191] and bistriazoloacridone [192] derivatives. The inhibitory effects of the fluoroquinolines [i.e. 8-difluoromethoxy-1-ethyl-6-fluoro-1,4-dihydro-7-[4-(2-methoxyphe-nyl)-1-piperazinyl]-4-oxoquinoline-3-carboxylic acid (K-12) and 7-(3,4-dehydro-4-phenyl-1-piperidinyl)-1,4-dihydro-6-fluoro-1-methyl-8-trifluoromethyl-4-oxoquinoline-3-carboxylic acid (K-37) (Fig. 16)] on the HIV-1 LTR-driven gene expression may at least in part be attributed to inhibition of Tat [193] but also other RNA-dependent transactivators [194]. The fluoroquinoline K-12 acts synergistically with reverse transcriptase and protease

inhibitors which means that such combinations may have potential as chemotherapeutic modalities for the treatment of HIV-1 infection [195].

Recently, a natural product isolated from *Streptomyces*, EM2487 (Fig. 16), was shown to inhibit HIV-1 replication at the transcription level, again through an action that, at least in part, could be attributed to inhibition of the Tat function [196].

The bistriazoloacridone temacrazine [1,4-bis(3-(6-oxo-6H-v-triazolo[4,5,1-de]acridin-5-yl)aminopropyl)piperazine] (Fig. 16) exerts its mechanism of antiviral action through a selective inhibition of HIV-1 transcription: the compound
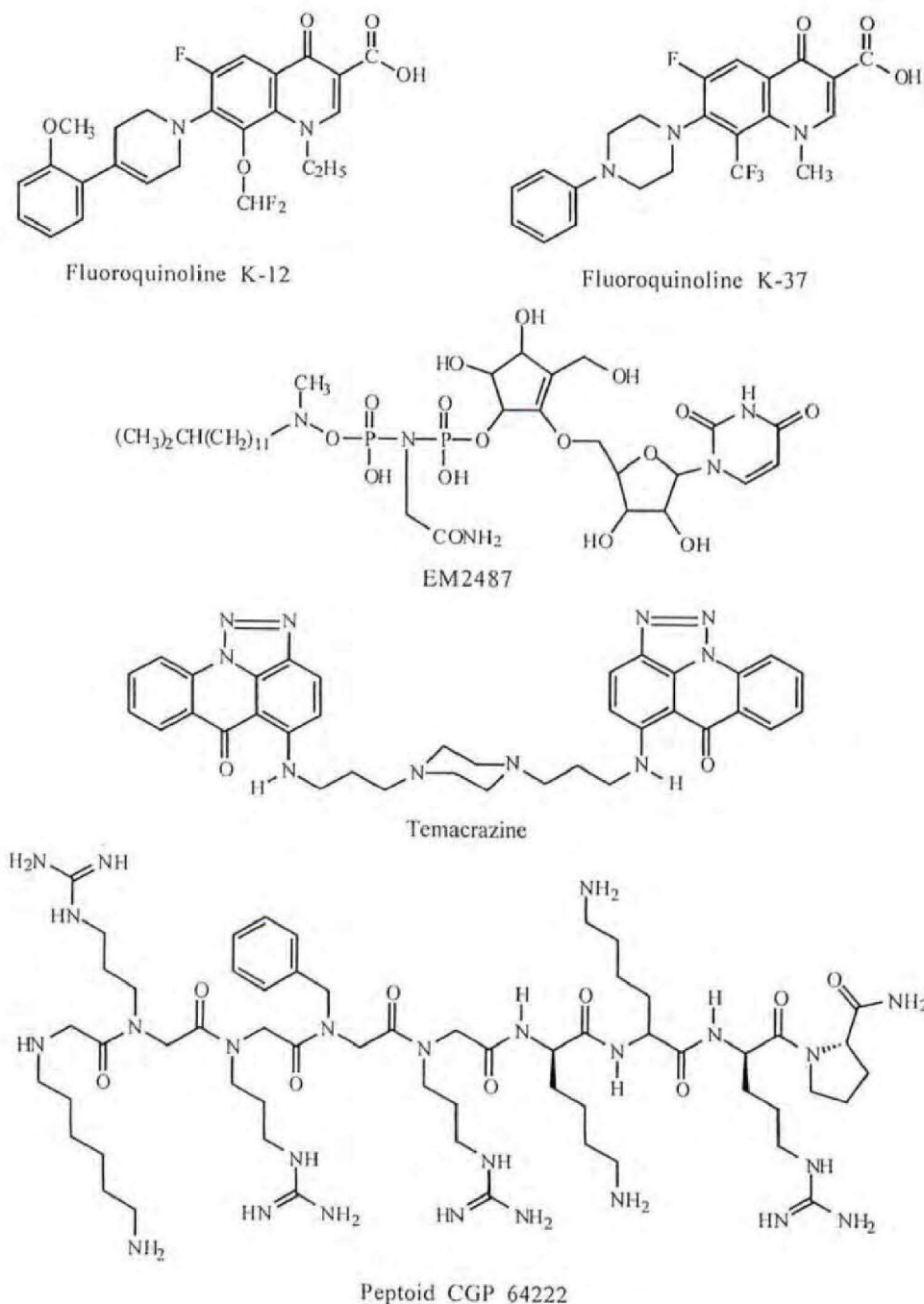

Fluoroquinoline K-12

Fluoroquinoline K-37

EM2487

Temacrazine

Peptoid CGP 64222

Fig. (16). Transcription transactivation inhibitors: Fluoroquinolines K-12 and K-37, *Streptomyces* product EM2487, Temacrazine and the peptoid CGP 64222.
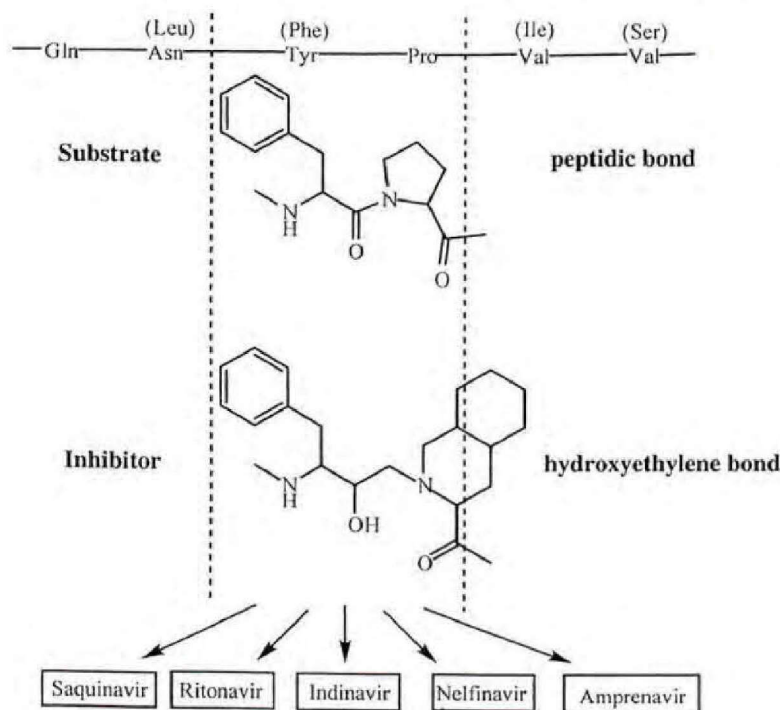
Fig. (17). Rationale of "peptidic" HIV-1 protease inhibitors: replacement of scissile peptidic bond by non-scissile hydroxyethylene bond.

was found to block HIV-1 RNA formation without interfering with the transcription of any cellular genes [192]. It inhibited HIV-1 replication in acutely, chronically and latently infected cells. Although temacrazine inhibited the 3' processing and strand transfer activities of HIV-1 integrase, no evidence of an inhibitory effect on the intracellular integration of proviral DNA into the cellular genome during the acute infection could be detected [192]. Resistant virus was generated by serial passage in escalating doses of temacrazine: greater than 1,000-fold resistance was achieved after six passages and genotypic analysis identified several unique nucleotide changes in the HIV-1 LTR at positions −1, −2 and +111 relative to the start of transcription. Thus, inhibition of HIV-1 replication by temacrazine could be reversed by LTR mutations increasing the basal rate of the viral transcript initiation [197].

Tat peptide analogs, encompassing the Tat core domain (amino acid residues 36-50) [198], or the basic domain (amino acids 48-56: RKKRRQRRR) [199] have been reported to inhibit HIV-1 replication (in acutely infected cells), and, as expected, these peptide analogs were able to effectively block the Tat transactivation process. The 9-mer peptoid CGP64222 (Fig. **16**), which is structurally reminiscent of the amino acid 48-56 sequence RKKRRQRRR of Tat, was also reported, on the one hand, to block the Tat/TAR interaction, and, on the other hand, to suppress HIV-1 replication (again, in acutely infected cells) [200]. We have demonstrated, however, that the peptoid CGP64222 owes its anti-HIV activity in cell culture primarily to an interaction with CXCR4, the coreceptor for X4 HIV strains [201], which is, perhaps, not surprising given the structural similarity of CGP64222 to the other, polypeptidic, CXCR4 antagonists T22 (Fig. **4**) [48], nona-

arginine (ALX40-4C) (Fig. **4**) [49], and T134 (Fig. **4**) [50]. In fact, Tat itself (following its extracellular release) has recently been shown to block CXCR4-dependent HIV-1 infection [202], presumably through blockade of CXCR4 by the above mentioned 48-56 amino acid portion (RKKRRQRRR) of the molecule.

HIV PROTEASE INHIBITORS

HIV protease inhibitors prevent the cleavage of the gag and gag-pol precursor polyproteins to structural proteins (p17, p24, p7, p6, p2, p1) and the functional proteins (protease, reverse transcriptase, integrase), thus arresting maturation and thereby blocking infectivity of the nascent virions [203]. The HIV protease inhibitors have been tailored after the target peptidic linkages in the gag and gag-pol polyproteins that have to be cleaved by the protease, *viz.* the phenylalanine-proline sequence at positions 167 and 168 of the gag-pol polyprotein (Fig. 17). All protease inhibitors that are currently licensed for the treatment of HIV infection, namely saquinavir, ritonavir, indinavir, nelfinavir and amprenavir [203,204] share the same structural determinant, i.e. an hydroxyethylene (instead of the normal peptidic) bond, that makes them non-scissile substrate analogues for the HIV protease (Fig. 17).

Also, the recently approved protease inhibitor lopinavir (ABT-378) (Fig. **18**), [204,205], follows the same principle, in that it acts as peptidomimetic inhibitor of HIV protease. Lopinavir is co-dosed with ritonavir at 400/100 mg twice daily. The reason for this combination is that ritonavir strongly inhibits the metabolism of ABT-378 and allows ABT-378 to reach much higher plasma drug levels upon oral

administration [206]. Also in phase III is atazanavir (BMS-232632), (Fig. **18**), which has been accredited with a favorable resistance profile, that does not parallel any of the individual protease inhibitors currently in use, as well as a favorable pharmacokinetic profile that would allow once-daily dosing [207]. This contrasts with another peptidomimetic protease inhibitor, KNI-272, that according to the initial phase I/II studies, would have rather unfavorable pharmacokinetics (short plasma half-life, extensive protein-binding, rapid elimination *via* the liver) [208].

Resistance mutations have been reported for most, if not all, peptidomimetric inhibitors of HIV protease. Some of these are unique for a certain protease inhibitor (i.e. D30N, which is the predominant amino acid substitution in patients treated with nelfinavir) [209], but others are common for most, if not all compounds (i.e. those at positions 20, 36, 46, 54, 63, 71, 82, 84 and 90 of the HIV protease) [204]. Obviously cross-resistance among protease inhibitors may be expected from such overlapping resistance patterns. This has prompted the search for new, non-peptidic inhibitors of HIV

protease, that, in addition to a broader anti-HIV activity spectrum, might also offer increased oral bioavailability and/or pharmacokinetic properties.

Examples of non-peptidic inhibitors of HIV protease include 4-hydroxycoumarins and 4-hydroxy-2-pyrones [210], sulfonamide-substituted derivatives [211], cyclic ureas (i.e. DMP 323 and DMP 450) [212,213] (Fig. **18**), cyclic cyanoguanidines [214], new aza-dipeptide analogues [215,216] and tipranavir (PNU-140690), a sulfonamide-containing 5,6-dihydro-4-hydroxy-2-pyrone [217-219] (Fig. **18**). Also, C_2-symmetric protease inhibitors with L-mannaric acid as the peptidomimetic scaffold [220] (Fig. **18**) and *N*-alkyl cage dimeric 4-aryl-1,4-dihydropyridines as non-peptidic HIV-1 protease inhibitors [221] have been recently reported.

The major advantage of the cyclic urea DMP 450 (mozenavir) is its substantial oral bioavailability observed in all species examined, including man [213]. DMP-450 has been the subject of phase I/II dose-escalating clinical studies and appears to have good antiviral activity and tolerability at

Lopinavir (ABT-378)

Mozenavir(DMP 450)

Atazanavir (BMS-232632)
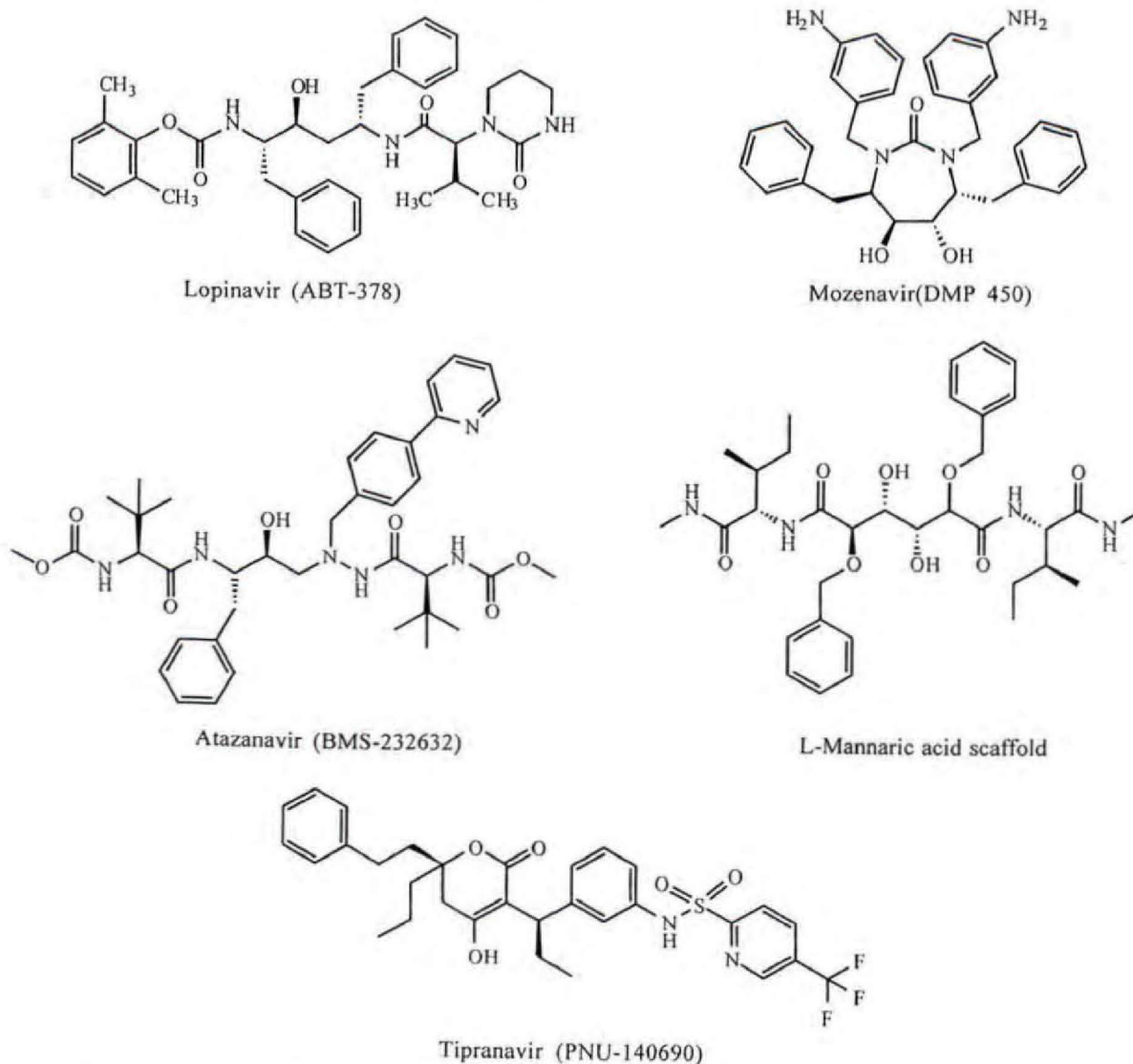
L-Mannaric acid scaffold

Tipranavir (PNU-140690)

Fig. (18). Newer inhibitors of HIV-1 protease: ABT-378, DMP 450, atazanavir BMS-232632, L-mannaric acid derivative and tipranavir.

all doses tested [222]. The new aza-dipeptide analogues (i.e., CGP 73547 or BMS-232632) combine excellent anti-HIV potency with high blood drug levels after oral administration; furthermore, BMS-232632 shows no cross-resistance with saquinavir-resistant HIV strains [215], and additive to moderately synergistic antiviral effects with other antiretroviral agents [216].

The most advanced among the non-peptidic inhibitors of HIV protease is tipranavir [224]. The drug can be synthesized by a convergent approach amenable to large-scale preparation in a pilot plant [217]; it inhibits HIV protease with a K_i value of 8 pM and inhibits HIV replication in cell culture at an IC_{90} of 100 nM [218]. *In vitro*, tipranavir showed low cross-resistance to HIV strains that were resistant to the established (peptidomimetic) inhibitors of HIV protease [219]. Also, tipranavir retained marked activity against HIV-1 isolates derived from patients with multidrug resistance to other protease inhibitors [223]. If tipranavir also shows little cross-resistance with other protease inhibitors *in vivo*, it may be a valuable addition to the armamentarium of antiretroviral agents [224], particularly for patients who have already failed other protease inhibitor-containing regimens [223].

It should be kept in mind that in addition to engendering resistance mutations in the enzyme itself, the use of protease inhibitors may also induce mutations at the *gag* proteolytic processing sites, i.e. at p1/p6 and p7/p1. This has also been encountered with ABT-378, where mutations at the *gag* proteolytic cleavage sites lead to the growth of highly resistant HIV-1 [225]. Such mutations may emerge in the presence of peptidic as well as non-peptidic protease inhibitors, since they give both rise to the same pattern of *gag* and *gag-pol* processing intermediate accumulation [226].

CONCLUSIONS

In recent years, an ever increasing number of compounds have been uncovered as anti-HIV agents targeted at virtually any step of the virus replicative cycle: adsorption, entry, fusion, uncoating, reverse transcription, integration, transcription (transactivation), and maturation. In addition to the "newer" NRTIs, NNRTIs and PIs, various other compounds, i.e. those that are targeted at viral entry (i.e. CXCR4 and CCR5 antagonists) and virus-cell adsorption/fusion (i.e. compounds interacting with either gp120 or gp41), offer great potential for the treatment of HIV infections. Most of the anti-HIV agents seem to accomplish their anti-HIV activity through an interaction with the presumed molecular target. However, quite a number of compounds are capable of interacting with more than one target so their true mechanism of action may not be what was expected. Three examples in point are the G-octet containing 17-mer oligonucleotide zintevir, the dicaffeoyltartaric acid L-chicoric acid, and the nonapeptoid CGP 64222. Zintevir and L-chicoric acid were originally identified as integrase inhibitors, and the nonapeptoid as a transactivation (Tat) antagonist, and their anti-HIV activity in acutely infected cells was ascribed to interference with the integration and transactivation process, respectively. As it

now appears, zintevir and L-chicoric acid primarily interact as virus adsorption inhibitors, and the nonapeptoid as a CXCR4 antagonist, and thus these compounds owe their anti-HIV activity mainly to interference with an early event (adsorption, entry) of the HIV replicative cycle. Caution should therefore be exercised in postulating from the alleged interaction of a certain anti-HIV agent with a certain viral protein (or enzyme) that the anti-HIV activity is necessarily due to this interaction [227,228]. The compound(s) may well be able to interact with other targets that are more crucial in the anti-HIV activity that is eventually achieved.

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