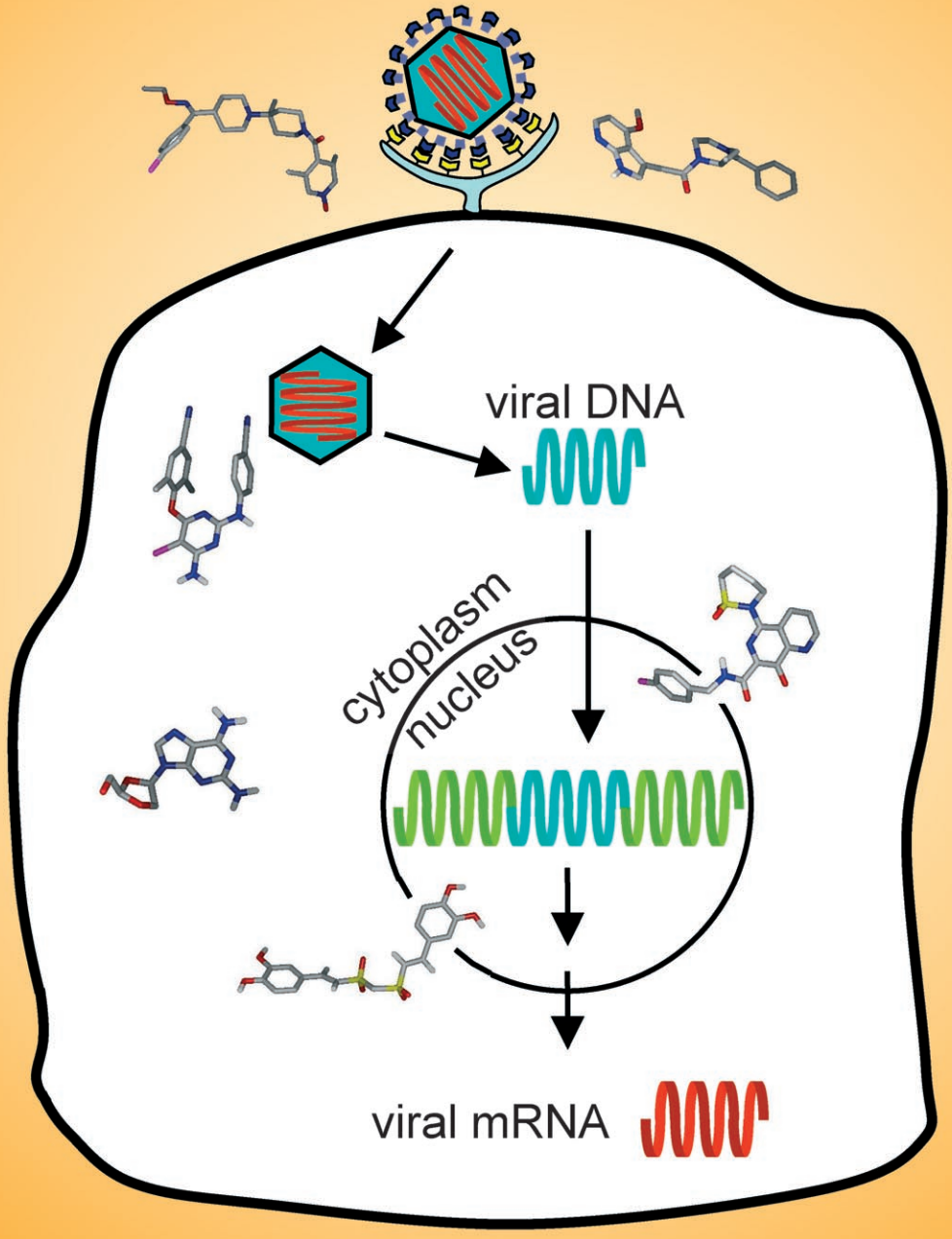
Targeting HIV



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Current Developments in HIV Chemotherapy

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HIV infection is the leading cause of death worldwide and despite major advances in treatment, more new cases were diagnosed in 2004 than any previous year. Current treatment regimens are based on the use of two or more drugs from two or more classes of inhibitors termed highly active antiretroviral therapy (HAART). Although HAART is capable of suppressing viral loads to undetectable levels, problems of toxicity, patient adherence, and particularly the emergence of drug-resistant viruses continues to spur the development of new chemotherapeutics to combat HIV. Clinical candidates from the four existing classes of inhibitors are presented in this review along with lead compounds against new viral targets, with special emphasis on HIV integrase.

1. Introduction

Since the first cases were reported in 1981, HIV has killed more than 20 million people worldwide, with approximately 39.4 million people living with the disease at the end of 2004.[1] With 4.9 million new cases reported in 2004, more than any year before, UNAIDS reports the epidemic is still in its beginning stages, as infection rates are exploding in high-risk populations in southeast Asia, particularly China, Indonesia, and Vietnam.[2] Despite major advances in treatment over the past 20 years, HIV has wreaked devastating socio-economic effects and caused tragic loss of life. Continued development of treatment options and vaccine research are of paramount importance to help stop the spread of this disease.

Currently, there are four FDA-approved classes of drugs to combat HIV infection: nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, and one fusion inhibitor. Initially, treatment for infected individuals consisted of a single inhibitor class: monotherapy. This had a modest impact on plasma HIV-1 RNA levels, but owing to the rapid emergence of drug-resistant virus strains and the inevitable development of serious immunodeficiency, monotherapy has become obsolete.[3,4] Consequently, combination therapy, which consists of three or more drugs from two or more different inhibitor classes, termed highly active antiretroviral treatment (HAART), was put into use. As a result, a drastic decrease in patient mortality has been observed.[5,6] Despite a dramatic increase in patient lifespan under HAART and successful suppression of HIV-1 RNA plasma levels for a prolonged period of time, the news has not been all good. Studies have shown that replication-competent viruses can be isolated from peripheral blood mononuclear cells and semen from infected patients who have maintained negligible plasma HIV RNA levels for up to two years; viral replication is incompletely suppressed using HAART.[7–9]

Other challenges facing HAART include drug resistance, patient adherence, and toxicity. Drug resistance is a consequence of incomplete suppression of viral replication. The high mutation rate of HIV causes mutations to accumulate, leading to diminished drug efficacy and gradually rendering each component of the regimen inactive.[10] These drug-resistant mutations can then be transferred through childbirth, sex, and needle sharing.[11] In fact, up to 10% of new infections are from virus strains resistant to 3′-azido-3′-deoxythymidine (AZT); half of these were also shown to be resistant to multiple classes of drugs.[12] Patient adherence is also an issue affecting resistant viruses. Whereas drug regimens are easier now than in previous years, they still must be taken twice a day with strict consideration to meal times for many years. Violating these regimens can lead to incomplete suppression and thus resistance.[13] Toxicity is another major concern in the administration of HAART and is yet another factor that influences adherence and thereby drug resistance. The propensity of these drugs to elicit such adverse side effects as severe lipodystrophy, glucose intolerance and diabetes mellitus, high cholesterol and triglyceride levels, and life-threatening cardiovascular complications has posed a significant barrier to treatment, leading to delays in the initiation of therapy.[14,15] Given these serious limitations, the ongoing search for new, milder, and more varied clinical treatment options is critical. Current preclinical or early clinical trials target almost all of the steps in the viral replication cycle, including virus adsorption (gp120), viral co-receptors (CCR5 and CXCR4), viral fusion (gp41), nucleocapsid protein (NCP7), transcription factors, and integrase.[16]

As previously mentioned, the viral replication cycle can be simplified into several discreet steps, each of which offers the potential for intervention by a therapeutic agent to help combat HIV infection. Infection begins with a specific interaction between the virion glycoprotein gp120 on the outer membrane and the CD4 receptor on the host cell surface (Figure 1, step a). In the next step, a conformational change occurs, allowing interaction of gp120 with chemokine co-receptor CXCRY or CCR5. Further conformational changes expose a fusogenic peptide, which then anchors into the host cell

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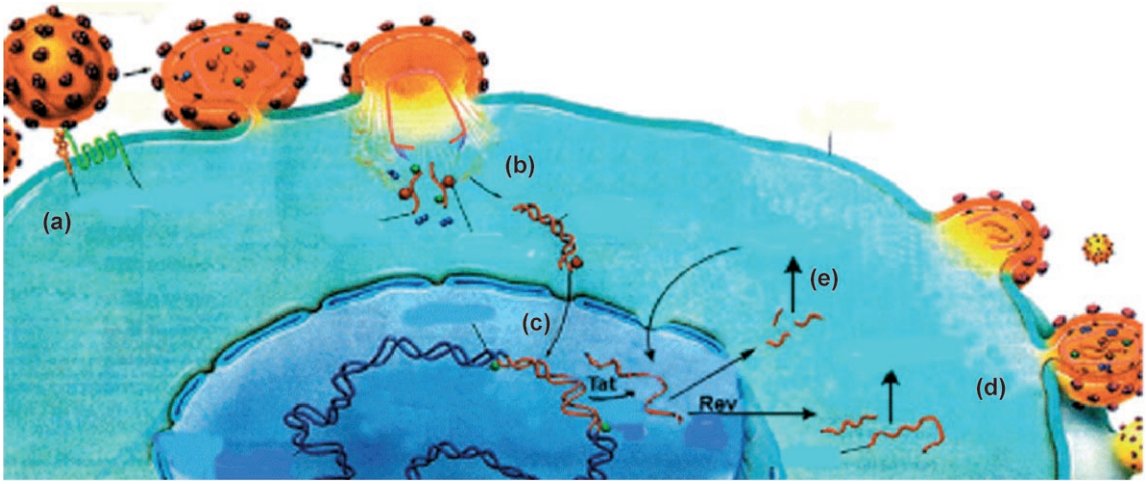

Figure 1. The HIV replication process (see text for details).

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membrane. Once the viral and cell membranes have fused, the virion uncoats, at which point the envelope proteins are lost, and a nucleoprotein complex is formed which begins the reverse transcription process (Figure 1, step b). Upon synthesis of the double-stranded DNA copy of the viral genome, the pre-integration complex is shuttled into the nucleus, where it is inserted into the host chromosome (Figure 1, step c). After transcription (facilitated by the regulatory proteins Tat and Rev, which are themselves viral gene products that return to the nucleus; step e) and translation of the viral DNA into viral proteins using cellular machinery, the assembly of the Gag and Gag–Pol polyproteins occurs near the cell membrane. During virion assembly, two copies of single-stranded viral RNA are incorporated into the virion, which then buds off the cell (Figure 1, step d), taking with it part of the host cell membrane. Soon after budding, viral protease cleaves the Gag–Pol polyprotein to render a mature, functional virion.[17]

2. Viral Entry

Viral entry is a complex process that can be divided into three steps: adsorption, co-receptor binding, and fusion. Each glycoprotein complex on the envelope of HIV-1 consists of a trimer of exterior gp120 glycoproteins and a trimer of transmembrane gp41 glycoproteins.[18,19] The entry process begins with an interaction between gp120 and the cellular CD4 receptor, at which point a conformational change occurs to permit a subsequent interaction with either cellular co-receptor CCR5 or CXCR4. The binding of gp120 to CD4 and a co-receptor then initiates the insertion of the hydrophobic N terminus of gp41 into the cellular membrane. The ectodomain of gp41 then forms a stable six-helix bundle, which consists partially of a trimer of coiled-coil heptad repeats (HR1). A second heptad repeat sequence (HR2) near the viral membrane forms an α helix that is complementary to the hydrophobic grooves of HR1. This collapse of the gp41 ectodomain brings the two membranes into close proximity, whereby fusion occurs (Figure 2).[18,20,21]

Each of these steps offers the potential for therapeutic intervention, and several approaches have been reviewed recently.[20,22] Only one drug from this broad class of potential targets has gained approval from the FDA: enfuvirtide (ENF) or fuzeon, a fusion inhibitor. ENF is a 36-residue synthetic peptide (YTSLIHSKIEESQNQQEKNEQELLELDKWASLWNWF), which contains a

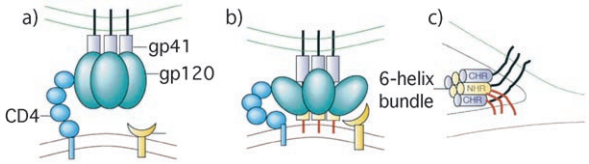


Figure 2. HIV adsorption and entry: a) attachment; b) co-receptor binding; c) fusion. (Reprinted from ref. [20] with permission from Elsevier, Ltd.)

sequence derived from a prototypic gp41 HR2 region. This sequence enables ENF to bind to the gp41 HR1 region, preventing the binding of viral HR2. The resulting malformed six-helix bundle precludes viral entry.[23] Though highly potent, ENF is currently used as a salvage therapy owing to its prohibitive production cost and low oral bioavailability. A second-generation fusion inhibitor, developed jointly by Roche and Trimeris, T-1249, contains a pocket-binding motif and shows higher antiviral activity, a longer half-life, and a different resistance profile than those of ENF.[18]

Whereas ENF is the only entry inhibitor to garner approval from the FDA, many others are in preclinical or clinical trials.[22] Small-molecule antagonists to CCR5 have demonstrated potent antiviral activity and good oral bioavailability with many diverse pharmacophores.[22] Figure 3 illustrates the most

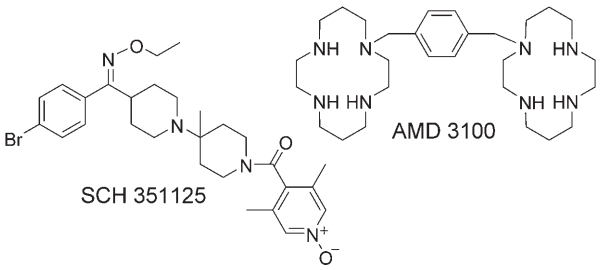


Figure 3. Representative examples of co-receptor antagonists.

advanced compound from this class.[24] Antagonists to CXCR4 have lagged behind those for CCR5; the most advanced, AMD 3100, was halted in clinical trials as a result of suboptimal efficacy.[25]

More recently, by conducting a synthetic SAR study of a protein, Hartley and co-workers identified an inhibitor of HIV entry (active in the picomolar range) that also provided protection from HIV infection in vivo.[26] Assembly of the protein RANTES (a natural ligand for CCR5) in two parts by using solid-phase synthesis allowed the rational substitution of both natural and non-natural amino acids (Figure 4). Through this process, the investigators were able to dissect the pharmacophore important for binding to CCR5. This elegant study represents the

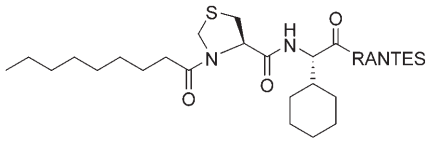


Figure 4. Structure of the most potent RANTES-based entry inhibitor, PSC-RANTES (IC_{50} = 36 pM). RANTES = *r*egulated upon *a*ctivation, *n*ormal *T*-cell *e*xpressed and *s*ecreted.

first application of modern medicinal chemistry toward a protein, and will undoubtedly lead to further SAR studies of biologically significant macromolecules.

Neffe and Meyer have also contributed to recent progress in viral entry inhibitors. Beginning with the crystal structure of gp120 bound to CD4, they started with the decapeptide sequence that comes into direct contact with CD4 and removed noncritical residues to decrease the molecular weight. They also incorporated non-natural elements to minimize the susceptibility of the peptide toward proteolytic cleavage. Ultimately, they identified a modified pentapeptide capable of binding to CD4 and blocking the gp120–CD4 interaction (Figure 5).[27]

Although considerable effort has been expended on the search for selective and potent HIV entry inhibitors, a majority

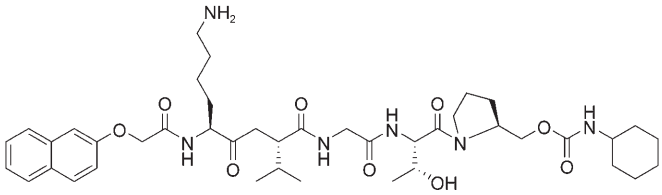


Figure 5. Structure of the peptidomimetic compound capable of CD4 binding (K_D = 35 μM).

of these are large peptides or peptidomimetic structures. More importantly, many act as antagonists for CD4 or the chemokine co-receptors. As mentioned above, many of these show a high degree of activity, but the long-term effects of blocking cellular receptors with important immune functions remain an important consideration. Finding a small-molecule HIV entry inhibitor that targets something other than the co-receptors, however, has met with relatively little success. One of the few successes with this task was reported by researchers at Bristol-Meyers Squibb who discovered a potent small-molecule entry inhibitor, BMS-378806 (Figure 6). This compound was found to inhibit a variety of HIV strains with a median EC_{50} of 12 nM. The inhibitor was shown to not interact with either chemokine co-re-

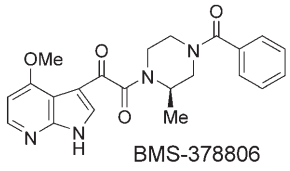


Figure 6. Structure of a small-molecule HIV-1 entry inhibitor.

ceptor, as demonstrated by its activity against cells naturally lacking co-receptors that were then transfected to express both CCR5 and CCR3. The authors determined that BMS-378806 interfered with gp120–CD4 interactions, but unlike the peptidomimetic designed by Neffe and Meyer, the small molecule bound to gp120 in a concentration-dependent manner and did not associate with CD4.[28]

Further investigations by Si and co-workers, however, found that BMS-378806 prevented infection by CD4-independent viruses of both CD4-expressing and CD-lacking cells, demonstrating that CD4 is not needed for antiviral activity. This suggested that the interference of gp120–CD4 complex formation is not the true mechanism of action for this compound. Through a series of complex antibody-binding and two-color flow cytometry experiments, the investigators suggested a model for BMS-378806 activity, in which the compound prevents CD4-induced conformational changes that expose the hydrophobic HR2 region of gp41 leading to the formation of the six-helix bundle.[29] The potent antiviral activity exhibited by many of the viral entry inhibitors clearly stands as a proof-of-principle for this class of drugs, however, considering the potential toxicity associated with the binding of host immune receptors and the cost of producing such large and complex structures as those required for inhibiting the more direct interactions between CD4 and gp120. Perhaps compounds that target intricate viral glycoprotein conformational changes have the best chances for progression into clinical use.

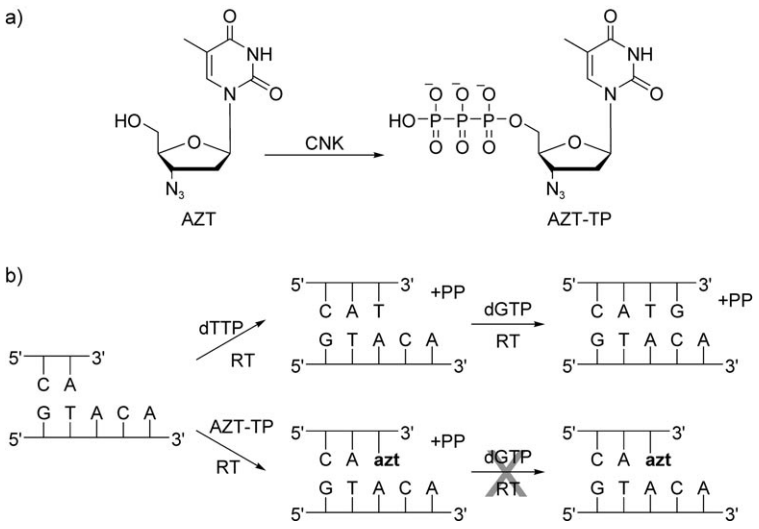


Figure 7. The NRTI AZT: a) AZT is a substrate for cellular nucleoside kinase (CNK); subsequent activity by nucleotide monophosphate- and diphosphate kinases yields AZT-triphosphate. b) AZT-TP can be incorporated into nascent DNA strands by HIV RT to abort transcription (bottom route).

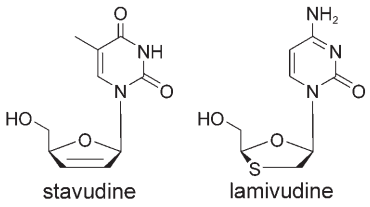


Figure 8. Example nucleoside reverse transcriptase inhibitors.

3. Reverse Transcriptase

Reverse transcriptase (RT) is the viral enzyme responsible for converting the single-stranded viral RNA genome into the double-stranded DNA form. Compounds that inhibit this enzyme are divided into two classes: nucleoside/nucleotide reverse transcriptase inhibitors (NRTI/NtRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). The two classes of inhibitors are discussed separately herein.

Nucleoside/nucleotide reverse transcriptase inhibitors

Compounds from this class of inhibitors represent those first used to treat HIV infection. These compounds are nucleoside analogues that lack a 3′-OH group so that once converted into the active triphosphate form and incorporated into the growing DNA strand, they terminate the polymerization process (Figure 7). Many functional groups have successfully been used to replace the 3′-OH group on the ribose ring, including an azide group in AZT, a C2′-C3′ double bond in stavudine, and replacement of the C3′ atom altogether with a thioether linkage in lamivudine (Figure 8); these drugs have been extensively reviewed.[30–32] Although the NRTIs can decrease the plasma levels of HIV RNA to an almost negligible quantity, toxicity toward mitochondria and the emergence of HIV strains with multiple drug-resistant mutations have been problematic for this family of drugs.[33,34]

The biggest challenge facing the design of new NRTIs is not in finding compounds that potently inhibit HIV, but rather in the identification of drugs with limited toxicity and, more importantly, little or no cross-resistance with other drugs. Attempts are being made at improving resistance profiles with such drugs as reverset, SPD 754 ((−)dOTC), and amdoxovir, all of which are in clinical trials (Figure 9). Reverset is a fluorinated cytidine derivative that has shown activity against HIV mutants resistant to lamivudine (M184V on RT) and AZT.[35] SPD 754 is another cytidine analogue that has proven effective against strains containing M184V and multiple thymidine analogue mutations; however, the administration of lamivudine in conjunction with SPD 754 significantly decreases the intracellular concentration of SPD 754 which leads to antagonistic drug activity.[36] The most advanced of the purine nucleoside analogues, amdoxovir (Figure 9), is deaminated intracellularly to dioxolane guanine through the action of adenine deaminase.[37] This drug is particularly attractive in that it retains activity against M184V, AZT-resistant mutants (M41L, D67N, K70R, T215Y, K219Q), and multiple drug-resistant strains from patients in whom treatment with other NRTIs had failed.[38]

NtRTIs are similar to NRTIs in mechanism of action, but contain a pendant phosphonate to decrease the number of intra-

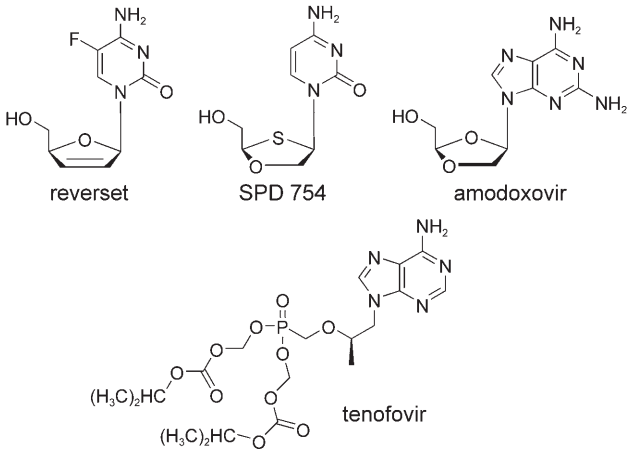


Figure 9. NRTI/NtRTIs in clinical trials or recently approved.

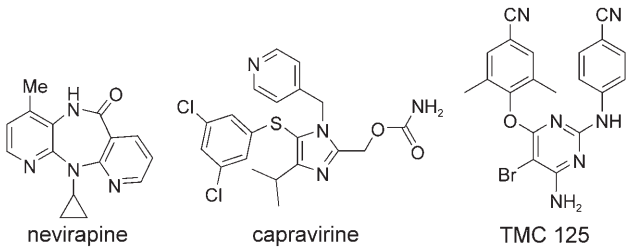


Figure 10. NNRTIs in clinical trials or approved.

cellular activation steps required for activity. Instead of the mandatory triple phosphorylation, compounds of this class require only two phosphorylation steps, which allows them to bypass the rate-limiting monophosphorylation step. One drug, tenofovir (Figure 9), has been licensed by the FDA and has become an important addition to antiretroviral treatment regimens. It was found that by adding tenofovir to the existing drug regimen for patients suffering NRTI-resistant infections, a significant decrease in plasma HIV RNA levels can be observed for up to 96 weeks.[39] These NRTI/NtRTIs have proven to be indispensable components of HIV chemotherapy, with every treatment regimen containing at least one member of this drug class. There are currently 12 NRTIs approved for use by the FDA.

Non-nucleoside reverse transcriptase inhibitors

It was first observed in the early 1990s that RT could be potently inhibited by compounds other than substrate analogues.[40] The NNRTIs encompass a structurally diverse set of compounds that inhibit RT by binding noncompetitively to an allosteric site near the substrate binding site (these compounds have been extensively reviewed).[41,42] The FDA has so far licensed three inhibitors of this class for the treatment of HIV infection: nevirapine (Figure 10), efavirenz, and delaviridine. It was observed, however, that treatment with the new class of inhibitors led to the rapid emergence of drug resistance. More worrisome was the fact that a single point mutation on RT (Y181C or K103N) often led to resistance to many different NNRTIs as a result of overlapping resistance profiles.[43]

A second generation of NNRTIs is currently being developed to deal with the problematic issue of drug-resistant mutations. The approach is to maximize interactions not with the amino acid side chains lining the allosteric binding pocket, but with main-chain atoms and conserved residues of RT such as W229.[44] Two drugs designed under the new paradigm entered phase II clinical trials: capravirine and TMC125 (Figure 10).

Capravirine has a greatly improved antiviral range over that of the first-generation NNRTIs and it is effective against viral strains that harbor the classic K103N and Y181C mutations.[45] Viral isolates collected from 52 patients who failed NNRTI therapy were treated with capravirine; 50% of the cases showed no loss of antiviral activity and 86% displayed an EC_{50} value less than 70 nM.[46] The impressive resistance profile of capravirine is probably the result of an extensive hydrogen-bonding network that includes main-chain residues of K101, K103, and P236.[47]

TMC125 also has a broader antiviral spectrum than first-generation NNRTIs, as it shows activity against the L100I, K103N, Y181C, and Y188L mutations.[48] Contrary to the hydrogen-bonding network exploited by capravirine, the improved resistance profile of TMC125 is thought to be attributable to the increased flexibility of the molecule which potentially gives it a greater number of binding modes in the active site.[49] Despite these attributes, clinical trials for capravirine were recently suspended due to poor performance and adverse effects with other therapies.

Since 1996, when the first NNRTI was licensed by the FDA, this class of drugs has played an important role in HAART. However, owing to problems of viral resistance, there has been no approval for the use of a new drug from this class since 1998, and at the time, there was a considerable amount of skepticism toward the viability of NNRTIs in the treatment of anything aside of wild-type HIV. Nevertheless, these anti-HIV agents hold great potential for drug discovery with their high selectivity, relatively low toxicity, and activities in the low nanomolar range. Recent findings that NNRTIs can target drug-resistant populations have renewed interest in this class of inhibitors.

4. Protease Inhibitors

The genome of HIV can be divided into three main genes: *gag*, *pol*, and *env*. The product of *gag* is a polyprotein of 55 kDa (Pr55gag) that contains the matrix, capsid, and nucleocapsid structural proteins in addition to several other peptides used in virion assembly and morphogenesis. The *pol* gene contains sequences for the three viral enzymes (RT, protease, and IN) that are translated as a polyprotein complex of 160 kDa, Pr160gag–pol. It is the job of viral protease (PR) to cleave the Pr55gag and Pr160gag–pol into individual, functional proteins,

and this step is required for the production of mature and infectious virions.[50] PR is a noncovalent homodimer that acts catalytically as an aspartic acid protease. The active site is located at the dimer interface and possesses a catalytic Asp residue from each monomer: D25 and D125.[51]

There are now eight PR inhibitors licensed by the FDA, making this class of compounds an integral part of HAART. The available PR inhibitors are all peptidomimetic transition-state analogues that contain a nonhydrolyzable transition-state isostere (Figure 11). Whereas highly active both in vitro and in the clinical setting, the peptide-based PR inhibitors have several disadvantages. These include the ever-present problem of drug resistance and cross-resistance. Moreover, many of these drugs have low oral bioavailability because of their peptide character, and they also possess the worst toxicity profile of all licensed anti-HIV drugs.

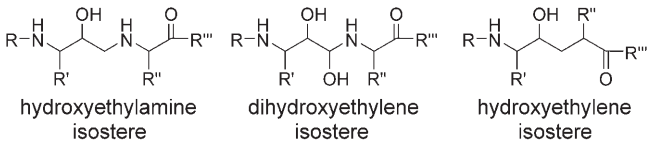


Figure 11. Transition-state isosteres in PR inhibitors.

Indinavir (Merck, Figure 12) is an example of the peptide PR inhibitors. Like most peptide PR inhibitors, indinavir has a high binding affinity for the PR of both HIV-1 and HIV-2 ($K_i = 0.34$ and 3.3 nM, respectively), antiviral activity in the nanomolar range ($ED_{95} \leq 100$ nM) and a synergistic profile with both classes of RTI.[52] However, Condra et al. found that multiple and variable mutations could cause phenotypic resistance to indinavir, and that all primary isolates of indinavir-resistant virus also showed resistance to ritonavir; 63% were resistant to saquinavir and 81%, to amprenavir, clearly demonstrating a pattern of cross-resistance in this class of HIV inhibitors.[53] Also concerning are the side effects, which include severe lipodystrophy, sharp increases in blood triglyceride and cholesterol levels, and insulin resistance. In fact, a recent study showed that lipodystrophy occurred in 60% of the patients receiving PR therapy after 13 months of treatment. By 21 months, 80% were afflicted, indicating PR inhibitors may not be as selective as originally thought; these drugs may target human aspartic acid proteases involved in lipid metabolism.[54]

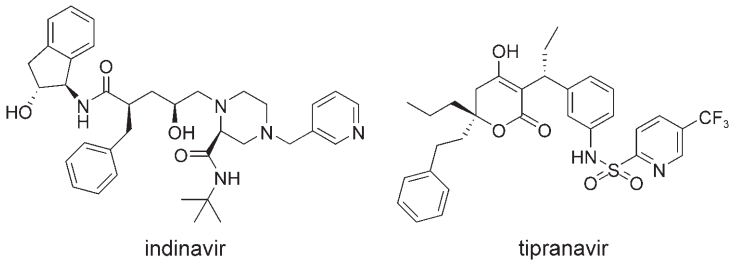


Figure 12. Peptidomimetic and non-peptidomimetic PR inhibitors.

Tipranavir is a potent PR inhibitor discovered through a process of screening and lead optimization of coumarins and coumarin-like compounds (Figure 12). It is important in that it was the first nonpeptide small molecule found to potently inhibit PR. Currently it shows promise in clinical trials, as it exhibits binding affinities in the picomolar and nanomolar ranges to the PR of HIV-1 and HIV-2, respectively. It has an EC_{50} value of 30 nM, and most importantly, it is fully effective against 90% of the viral isolates resistant to other PR inhibitors.[55]

Whereas an intense search for small-molecule PR inhibitors continues for the purpose of to combating resistance, another recent approach has been the use of peptidomimetic structures—not to bind the active site, but rather to bind to the dimer interface and prevent the dimerization process (Figure 13).[56] Although this approach is still in its infancy, it has been particularly successful. Peptidomimetics have been identified with binding affinities in the nanomolar range, and as the dimerization region is relatively conserved, the activity against PR with multiple drug-resistance mutations has been maintained.[57] PR inhibitors are a mainstay of modern retroviral therapy despite their notorious toxicity problems. With ongoing research, especially in the area of rationally designed small molecules and the targeting of novel portions of the protein that are more impervious to mutation, this class of drugs will continue to play a major role against HIV infection.

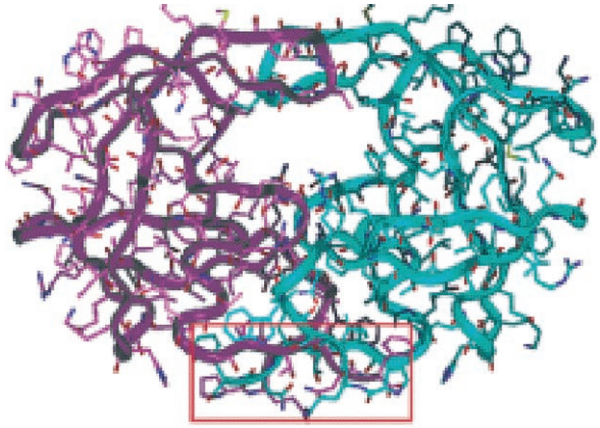


Figure 13. HIV-1 protease; the dimer interface region is boxed.

5. Integrase

Integrase (IN), the third of the viral enzymes, is arguably the most promising of the new targets in preclinical or early clinical trials. IN is a protein of 32 kDa responsible for the insertion of newly reverse-transcribed double-stranded viral DNA into the host genome where it can then be replicated using host cell machinery during transcription or cellular replication. What makes IN an especially attractive target for drug design is that

there are no known mammalian counterparts to this enzyme, so toxicity is expected to be rather low. Furthermore, integration is an essential part of the viral replication cycle, and the IN region of the *pol* gene is more conserved than either the RT or PR coding regions.[58,59] This enzyme performs two catalytic functions: 3′-end processing and strand transfer or integration.[60] In the cytoplasm of the cell, IN binds to double-stranded viral DNA as it is being reverse-transcribed to form a preintegration complex. It then cleaves two terminal nucleotides from each 3′ end of the DNA in a conserved CA-rich region (3′-end processing). The preintegration complex is then shuttled into the nucleus, where the recessed 3′ ends of the viral DNA are directly inserted into the cellular DNA phosphodiester backbone through a pair of transesterification reactions (strand transfer or integration). The two overhanging 5′-end nucleotides are cleaved, and the backbone is then ligated to render cellular DNA containing the viral genome (Figure 14).[61]

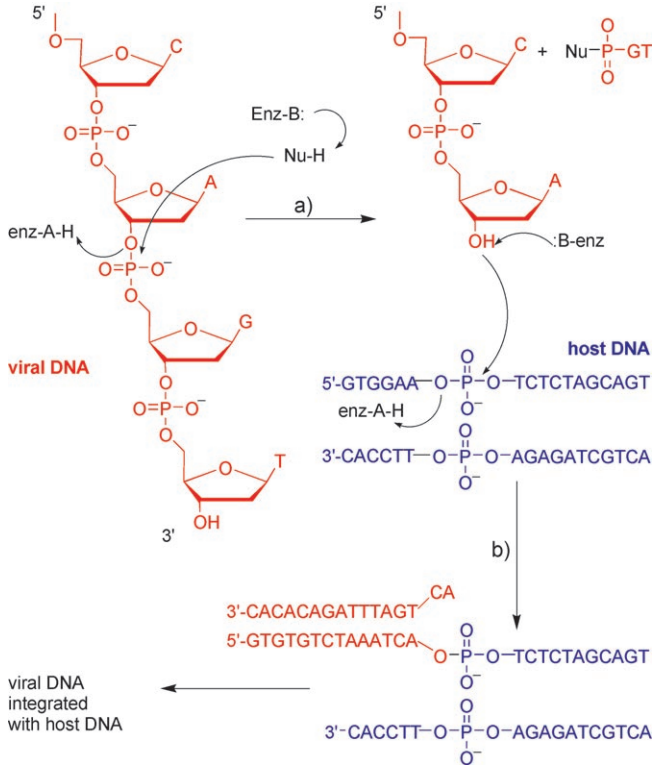


Figure 14. Reactions carried out by IN: a) 3′-end processing and b) strand transfer.

IN can be divided into three distinct domains: a Zn-binding N-terminal domain, a catalytic core domain, and a DNA-binding C-terminal domain.[62] The catalytic domain contains the highly conserved DDE motif characteristic of all integrase proteins and also found in DNA transposases and retrotransposases. These three conserved residues have been found to bind a divalent metal cofactor (either Mg^{2+} or Mn^{2+}) and through point mutation studies, have been found critical for enzyme activity.[63] The structures of all of the HIV-1 IN domains have been solved by NMR spectroscopy and/or X-ray crystallography either individually or in pairs, but solution of the complete structure containing all three domains has remained elusive.[64–67] It has been shown, however, that purified integrases from a variety of retroviruses can be present in dimeric, tetrameric, or oligomeric form in solution, and that HIV IN is present in equilibrium between dimeric and tetrameric forms in solution.[68–70] Despite the confusion over the degree of multimerization, more recent studies indicate that a tetramer (a dimer of dimers) is probably required for the full integration reaction, with two of the active sites bound to DNA and the other two serving as structural support.[26] Another point of interest is the possibility of a second divalent metal ion in the active site. This theory has been proposed and generally accepted for a number of DNA polymerases.[71,72] Although the presence of a second metal ion has not been observed in the X-ray crystallographic studies, it is reasonable to suggest that the second metal ion enters the IN active site cooperatively with the DNA; to date, there is no co-crystal structure of IN with DNA. The Mg^{2+} ion that does appear in the crystallographic data is coordinated between Asp 64 and Asp 116, and Neamati and coworkers have speculated that the second metal ion is coordinated by Glu 152 and possibly Asp 64.[73] In addition to these two major unanswered questions regarding multimerization and the second metal ion, the location of a highly flexible loop that overhangs the active site (residues 139–152) and other structural inconsistencies have been significant obstructions to the search for anti-HIV chemotherapeutics targeting IN.

The first compounds found to inhibit IN were generally charged and/or nonspecific. One of the first compounds reported to inhibit IN in vitro is the aurintricarboxylic acid monomer (**1**, Figure 15).[74] This compound was also found to inhibit RT and other phosphoryltransferases. It is therefore not a good drug candidate, but it did inspire the scientific community to consider IN as a target for drug design. A related class of compounds known as the cosalane analogues, exemplified by cosalane (**2**, Figure 15) contain a steroidal core with a disalicylmethane appendage. These compounds were capable of inhibiting IN activity in the micromolar range, but the antiviral effects were caused mainly by interference with the initial gp120–CD4 interaction.[75] Another major class of early IN inhibitors includes DNA binders and topoisomerase inhibitors such as doxorubicin and mitoxantrone (**3** and **4**, respectively, Figure 15). These were not very promising as potential therapeutics owing to weak and nonselective inhibition.[76,77]

It has also been shown that a wide variety of flavonones and related compounds exemplified by quercetagetin (**5**, Figure 15) inhibit IN. SAR studies have been conducted for this class of molecules, and demonstrate a number of structural features that are important for activity: 1) at least three hydroxy groups are required for activity, 2) inhibition is proportional to the number of *ortho*-hydroxy group pairs, 3) activity is abolished with the presence of glycosyl or methoxy substituent(s), and 4) activity generally increases with increasing number of hydroxy groups.[78] Whereas the flavones show potent enzyme inhibition, they are notoriously nonspecific and inhibit ion-transporting ATPases, topoisomerase II, and a wide range of DNA and RNA polymerases.[37]

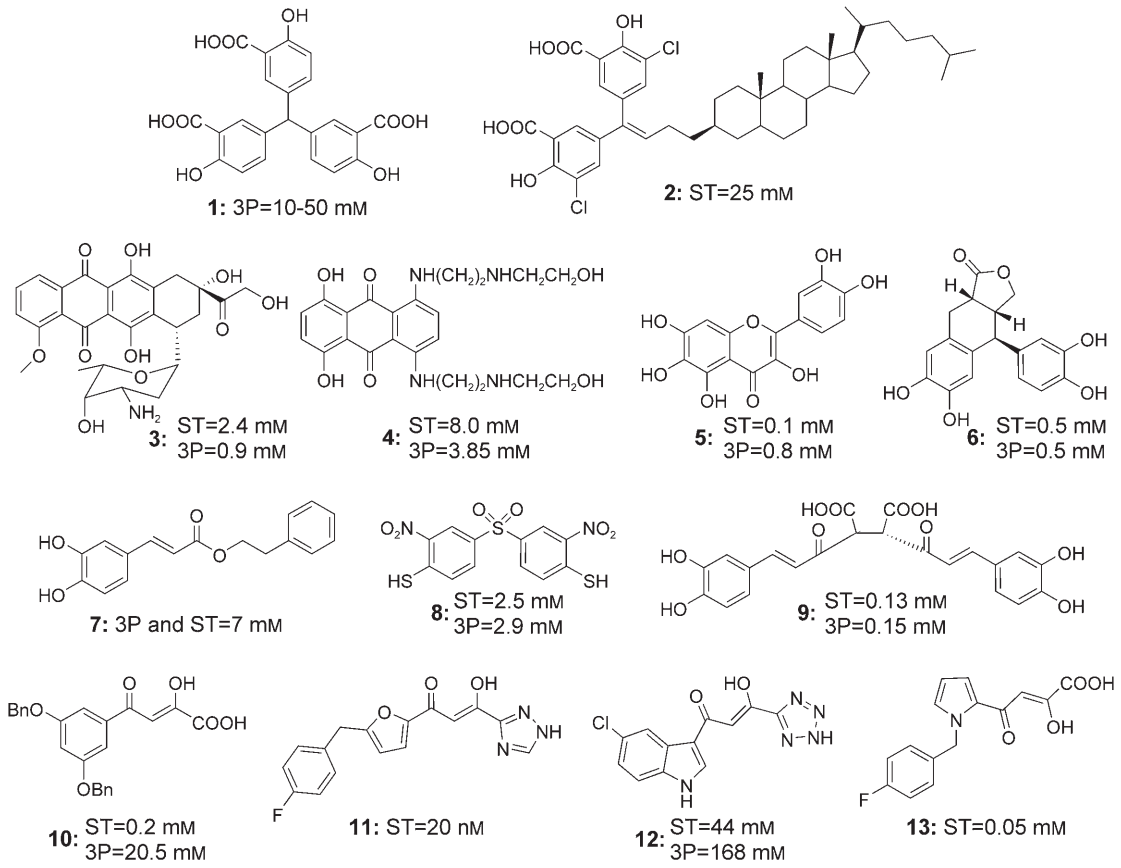


Figure 15. Reported inhibitors of HIV-1 IN; IC_{50} values: ST = strand transfer (integration), 3P = 3′-end processing.

In 1995, the bis-catechol pharmacophore was introduced as a member of the first selective inhibitors of IN.[79] β-Conidendrol (**6**, Figure 15) and its analogues were identified as potent inhibitors of IN, with in vitro enzymatic assays giving inhibition constants of 0.5 μM. Although none of the compounds tested had antiviral activity, these findings were still important, as these were the first inhibitors to be selective for IN; they did not affect functionally similar enzymes such as the restriction endonuclease Eco RI, HIV-1 RT, HeLa RNA polymerase II, mammalian topoisomerases I and II, or the influenza virus polymerase–endonuclease complex. The investigators found that replacement of the hydroxy groups with methoxy substituents substantially decreased potency, and removal or masking (as the methyl ether) of one of the *ortho*-hydroxy group pair significantly diminished activity. Another SAR was conducted on a simplified flavonoid variant known as caffeic acid phenylethyl ester (CAPE, **7**, Figure 15) and similar results were found.[80] Unlike the flavonoids, **7** and its related analogues displayed preferential inhibition of IN. As was the case for the SAR studies of the flavonoids and **6**, it was found that the nature and placement of ring substituents on **7** are important factors for activity. Similarly, replacement of hydroxy groups by methyl ethers or fluorine atoms resulted in a loss of activity, as did alterations to the *ortho*-hydroxy motif. However, the potency of inhibition was increased by the addition of a third hydroxy group in either the 2 or 5 position. The presence of the phenyl ester was also investigated and found to be important for activity, as both the methyl and ethyl esters were inactive. Lastly, the central ester was found to be important for activity, as the amide analogue was inactive. Whereas the bis-catechol was selective and potent, it was also plagued with problems of toxicity. In the SAR study of CAPE (**7**), low therapeutic indices were noted for all compounds that were active in cellular studies owing to the high level of toxicity.[39] This toxicity is not altogether unexpected upon consideration of the nature of the *ortho*-hydroxy group pair.

Polyhydroxylated styryl compounds are well-known inhibitors of protein-tyrosine kinase (PTK), with a 1,2- or 1,4-dihydroxy motif generally required for activity.[81,82] The nature of this activity is the result of the two sequential one-electron oxidations of a 1,2- or 1,4-dihydroxy motif to the *ortho*- or *para*-quinone, respectively. This reactive species can then undergo two separate 1,4-Michael-type additions by biological nucleophiles to result in protein cross-linking and cytotoxicity (Figure 16).[83] This oxidative mechanism, therefore, may be the mode of action for the bis-catechol compounds. There is one

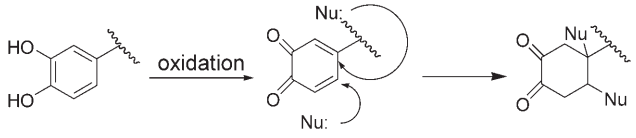


Figure 16. Potential oxidative addition mechanism in which biological nucleophiles attack a 1,2-dihydroxy motif to give an *ortho*-quinone product.

piece of evidence that does not support this hypothesis, however; an alternate mode of action is supported by the fact that compounds with an easily oxidizable *para*-dihydroxy motif are inactive.

Diaryl sulfones (general structure exemplified by **8**, Figure 15) were investigated as potential inhibitors of IN in 1997.[84] This class of compounds was investigated because it was found that some diaryl sulfones inhibit HIV RT, and some pyrrolyl aryl sulfones were antiviral yet inactive against RT, thus implicating an alternate step in the replication cycle.[85] Taken together with the fact that sulfones are well-documented as antibacterial, antimalarial, and antileprotic agents, these were thought to be a good starting point for a new class of potential IN inhibitors.[86,87] The investigators tested two broad classes of diaryl sulfones based on the electronic nature of the substituents: electron-withdrawing versus substituents in the *ortho* or *meta* positions, most of which were electron-donating. In the first set, it was found that one hydroxy group of the catechol could be replaced by a nitro group, while the other could be replaced with a strong electron-withdrawing group and retain activity. Also, the replacement of a hydroxy group by a thiol resulted in increased potency. In the second set of compounds, many of the substituents chosen were very reactive aldehydes or charged acid groups. Whereas the study presented a potential new class of IN inhibitors, it did not result in any promising candidates owing to either cellular toxicity, active IN inhibition with no observed antiviral effects, or antiviral activity without IN inhibition, which implicated an alternate mechanism of activity.[38]

Some very promising IN inhibitors were identified in 1996. The dicaffeoylquinic acids (DCQAs) come from the traditional medicines of the Bolivian Kallawaya culture and have a rich ethnopharmacologic history. Robinson and co-workers tested two natural DCQAs, three likely DCQA precursor molecules, and a synthetic analogue, L-chicoric acid (**9**, Figure 15).[88] Whereas none of the three precursor molecules, caffeic acid, quinic acid, or chlorogenic acid showed inhibition, both the natural DCQAs and the synthetic L-chicoric acid (**9**) proved to be potent inhibitors, with **9** showing the highest potency at approximately 1.1 μM and 0.8 μM for 3′-end processing and integration, respectively.[38] Like the flavonones, chicoric acid and DCQA analogues have been intensely studied and have been the subject of many SAR studies.[89,90] In these studies, it was shown that 1) both enantiomers of chicoric acid are equally potent inhibitors, 2) inhibition is maintained in the absence of the central carboxylic acid moieties as long as the catechol groups are present, 3) derivatization of the catechols as methyl ethers abolished activity, 4) if the catechols are derivatized as the acetates, the presence of at least one carboxylic acid group is required for activity, and 5) activity is retained with the central acid(s) masked as the methyl ester.[40]

Although chicoric acid and its analogues were very promising inhibitors of IN, showing both specificity and a high degree of potency against the enzyme and in cellular assays, the use of these compounds as leads for drug development was reconsidered in 2000 when it was reported that IN was the cellular target of neither chicoric acid nor its acetylated analogues.[91] The study concluded that owing to the low membrane permeability of chicoric acid, the major antiviral effects of these compounds resulted from the interference of the initial gp120–CD4 interaction. The investigators proved this interaction to be the target of chicoric acid by three different methods. First, strains of HIV that are resistant to drugs known to interfere with viral entry (DS 5000, the G-quartet AR 177, and the bicyclams AMD 2763 and AMD 3100) were selected. The activity of chicoric acid was tested against these strains, and it was observed that its inhibitory capacity toward IN was decreased. A similar resistance profile to other gp120-targeted compounds suggests a similar mode of action. Second, time-of-addition studies were performed to determine how long after infection the administration of a test compound can be delayed while maintaining inhibition. Typically, the addition of entry inhibitors can be delayed by only 1 h after infection, reverse transcriptase inhibitors may be delayed 4 h, and protease inhibitors, for 18–19 h before antiviral activity is lost.[92] In this study, the addition of chicoric acid could not be delayed for over 1 h after infection, indicating that the compound interferes with an initial binding event. Lastly, a chicoric acid-resistant HIV strain was generated after passing the virus through increasing concentrations of chicoric acid. Pro-viral DNA was isolated and sequenced to reveal several mutations in the gp120 gene; no significant mutations were present in the IN gene. This result provided the strongest evidence for chicoric acid as a gp120 antagonist. It is well known that anionic compounds interfere with gp120 binding, probably through interaction with the polycationic V3 loop.[93] Therefore, any anionic compound that targets IN should be thoroughly tested to preclude the possibility of inhibiting an earlier step in the replication cycle. It should be noted that the above-listed classes of IN inhibitors are only a very small fraction of compounds known to inhibit IN. A large number of compounds have shown anti-IN activity in enzymatic assays, but *the vast majority of these do not exhibit antiviral activity in cellular assays*.[94,95] This, combined with the ambiguous targets of compounds that do display antiviral activity, has made the search for new IN inhibitors anything but trivial.

The independent identification of the diketo acid pharmacophore in 2000 by researchers at Merck and Shionogi & Co. brought with it a renewed interest in the search for IN inhibitors.[96] After screening more than 250 000 compounds, it was found that most of the inhibitors fell into this diketo acid category and were specific inhibitors of the strand-transfer or integration steps. The investigators also found that these were the most potent inhibitors described: the representative compound L-708,906 (**10**, Figure 15) has an IC_{50} value of 150 nM against strand transfer and antiviral activity in the range of 1–2 μM. As these compounds possess a carboxylic acid group, it was necessary to prove that IN was indeed the target and not interference with virus binding and fusion, as was the case for the chicoric acid analogues. To do this, the researchers selected variants resistant to **10** and sequenced the cDNA from the population. T66I, S153Y, and M154I mutations were detected in every clone, and because all of these residues are proximal to the catalytic Asp 64 and Glu 152 residues, it can be conclud-

ed that the diketo acids bind in or near the IN active site. Since their introduction, this class of compounds has received more attention than any previous class of IN inhibitors, with at least one drug, S-1360 (**11**, Figure 15), progressing into clinical development.[97]

The first crystal structure of an inhibitor complexed with the catalytic core of IN was reported with a drug designed by Shionogi & Co., 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2*H*-tetrazol-5-yl)-propenone (5CITEP) (**12**, Figure 15).[98] This shows a clear interaction between the inhibitor and the middle of the active site between the catalytic triad. The tetrazole ring of **12** also interacts with Lys 156, Lys 159, and Gln 148, all of which have been shown to be crucial for binding viral DNA.[99] More mechanistic information was obtained from studies conducted by Hazuda and co-workers on diketo acid L-731,988 (**13**, Figure 15) which was incubated with IN over various times and with seven different concentrations of target DNA.[100] A consistent loss of inhibition was observed with increasing concentrations of target DNA; loss of inhibition was also observed if target DNA was added prior to the addition of the inhibitor, with a 100-fold decrease in potency if the target DNA is added 30 min before addition of the inhibitor, relative to concurrent addition. These data suggest that target DNA and the inhibitor occupy the same region of the active site. Another interesting observation from this study is that the inhibitor bound with 1000-fold higher affinity to the strand-transfer complex (IN combined with viral DNA ends) than that to the noncomplexed enzyme, indicating a specific conformation of the active site is required for efficient inhibitor binding. This conformational change occurs when viral DNA is bound to the enzyme.

Multiple SAR studies have been conducted on this class of IN inhibitors. These studies have shown that the carboxylic acid group is required for activity in cell culture. Compounds like 5CITEP (**12**), which are equipped with the isosteric tetrazole ring, lack cellular activity.[101,102] With analogues of **13**, it was found that the intrinsic activity correlates with the angle between the benzyl and diketo moieties: potency increases as the angle increases from 60° to 118°.[103] This study also showed that a change in the number or nature of substituent(s) on the distal benzyl ring had little or no effect on activity. Conversion of the central pyrrole ring to a benzene ring and the addition of a methoxy substituent to the 3 or 4 position (with the butanoic acid group designated at position 1) led to a decrease in activity, whereas the addition of a methoxy, ethoxy, or isopropoxy substituent at position 2 led to a significant improvement in potency. Concerned about the viability of the critical 1,3-diketo acid functionality and seeking neutral inhibitors of IN, researchers at Merck identified a replacement for the biologically labile 1,3-diketoacid in 2003.[104] Based on a set of hypotheses regarding the structure of the 1,3-diketoacid, a new structure was designed in which the pharmacophore was kept intact by masking it in a less labile form: 8-hydroxy-[1,6]-naphthyridine. This maintains the enol form of the 1,3-diketone by incorporating the enol into a naphthyridine ring system and then using one of the heteroatomic nitrogen atoms of the naphthyridine as a carboxylic acid isostere. This not only makes the pharmacophore more robust, but also retains coplanarity of the ring systems. Under this new system, a compound equally as potent as the representative diketo acid was identified, but with a much better pharmacokinetic profile.

There had been much debate as to whether IN is a realistic drug target owing to the relatively limited time it remains active in the viral replication cycle. Skeptics felt that there would not be sufficient concentrations of drug in the active site region to block activity of the enzyme. Over the past four years, researchers at Merck have provided an increasingly convincing argument that IN is a truly valuable target against HIV infection. Recently, Hazuda and co-workers demonstrated a sustained suppression of viremia in rhesus macaques that were challenged with simian–human immunodeficiency virus (SHIV) and subsequently treated with IN inhibitor L-870,812 (**14**, Figure 17), a compound of the 8-hydroxy-[1,6]-naphthyri-

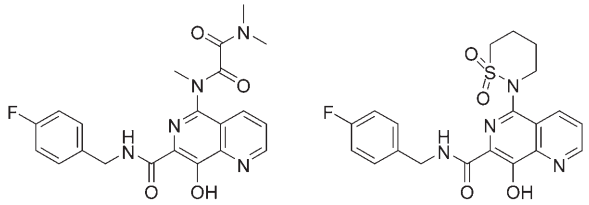


Figure 17. Potent inhibitors of HIV-1 IN; ST = IC_{50} values for strand transfer (integration).

dine class.[105] They also showed that serial passage of HIV-1 in cells in the presence of a related inhibitor, L-870,810 (**15**, Figure 17), caused diminished drug activity resulting from the accumulation of mutations in the IN-encoding region. This further supported the fact that drug resistance is not limited to RT and PR inhibitors, but that resistance is an issue regardless of the target.[106] An encouraging result from the identification of mutants, however, was that the major mutant isolated (N 155 H) demonstrated a decreased replication capacity and lower pathogenicity, suggesting a higher genetic barrier to resistance. Notably, the investigators compared **15** with **13** and found that by varying substituents on the required pharmacophore, a different resistance profile was observed, indicating that by changing the substituents on a fixed pharmacophore it may be possible to obviate problems of drug resistance.

The Gervay-Hague research group has been interested in the synthesis of sulfones as neutral phosphate mimics for use in a variety of enzyme inhibitors.[107,108] This is not the first time sulfones have been proposed as phosphate isosteres. Indeed, Benner and co-workers have studied the physical and chemical effects of sulfone analogues of oligonucleotides extensively.[109,110] In contrast to the approach of Benner, Gervay-Hague et al. have used a unique disulfone/diphosphonate reagent **16** (Figure 18) to synthesize a large number of highly functionalized geminal disulfone-containing compounds through Horner–Emmons–Wadsworth (HEW) olefination chemistry.[111] The idea comes from the use of a methylene unit as a replacement for the bridging oxygen atom, thus rendering the phos-

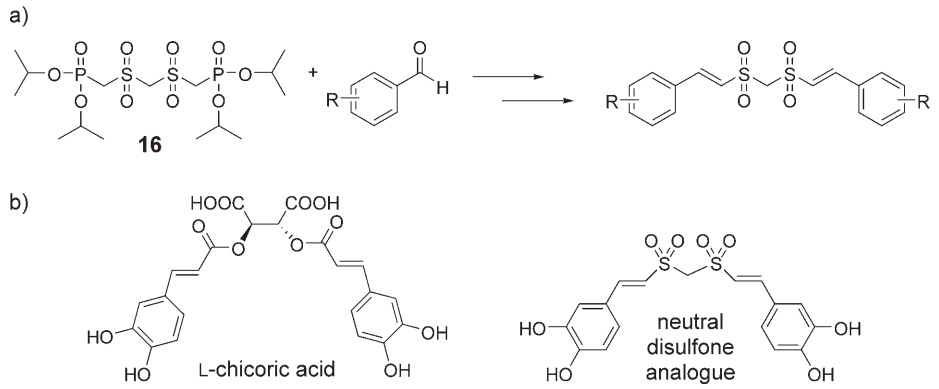


Figure 18. a) A unique disulfone/diphosphonate reagent **16** for HEW chemistry and b) example chicoric acid-like structures.

phate isostere nonhydrolyzable and replacement of the phosphorous atom with sulfur and oxidizing it to the sulfone to increase metallophilicity. Taken together, these design principles 1) maintain the correct bond length found in a phosphate backbone, 2) remove the chirality of the natural phosphate, thus simplifying isolation, synthesis, and characterization, and 3) maintain a nonionic compound, and provide a similarly metallophilic group. Because the pK_a value of the methylene bride between the sulfone groups is known to be 12.5, the compounds based on this scaffold are sure to be neutral in a biologically relevant environment.[112] Based on the fact that IN is a metal-containing enzyme responsible for making and breaking bonds of the DNA phosphodiester backbone, sulfone-based phosphate isosteres seem particularly well-suited for potential inhibitors of this enzyme, especially considering that both natural and non-natural dinucleotides are known inhibitors of IN, and the structures obtained through the disulfone/diphosphonate reagent could be remarkably similar to the chicoric acid class of IN inhibitors, depending on the choice of aldehydes used.[113]

Meadows et al. used this methodology to synthesize a library of *gem*-disulfone-containing chicoric acid analogues.[114] Indeed, of the ten analogues synthesized, eight were identified as moderate to highly potent inhibitors of IN (Table 1). Moreover, the compounds were found to possess potent antiviral activity in cell culture.

Most recently, Japan Tobacco has patented nitrogenous-condensed ring-containing compounds that target HIV IN (Figure 19).[115] These compounds, currently in phase I clinical trials in Japan, were recently licensed to Gilead Sciences for further drug development.[116]

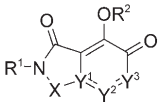
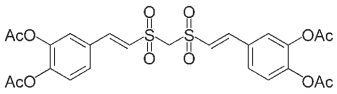
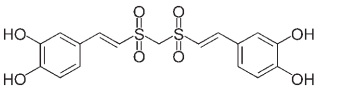
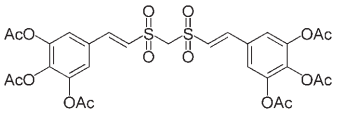
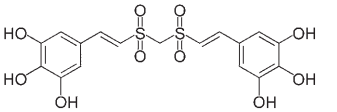
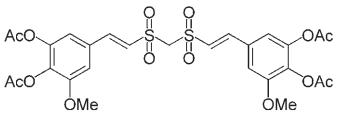
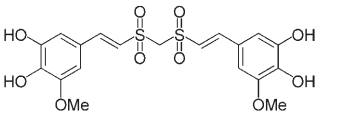
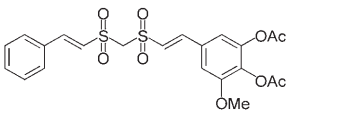
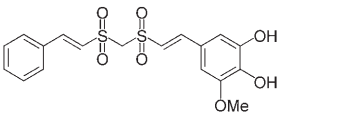


Figure 19. General structure of an IN inhibitor developed by Japan Tobacco. X = C,N perturbations, Y^n = C,N perturbations.

6. Summary and Outlook

Whereas drugs that target two of the three viral enzymes (RT and PR) have been in use for more than ten years, the development of a drug against the third viral enzyme (IN) has not yet been carried out. It has been hotly debated whether IN represents a viable drug target, as its period of activity is relatively short in the replication cycle of the virus. Moreover, the wide, shallow active site of IN and the unclear three-dimensional organization of its domains in vivo present formidable challenges to the design of drugs against this enzyme. However, much progress has been made over the last five years, and lead candidates are beginning to enter clinical trials with promising out-

Table 1. Activities of some disulfone-containing analogues of chicoric acid.

Compound	$IC_{50} \pm SE$ [μM][a]	$EC_{50} \pm SE$ [μM][b]
	70 ± 5	8.0 ± 2.5
	5 ± 1	2.4 ± 0.3
	5 ± 2	0.4 ± 0.1
	0.9 ± 0.1	0.3 ± 0.03
	30 ± 2	1.5 ± 0.02
	50 ± 10	1.3 ± 0.09
	80 ± 10	3.9 ± 1.2
	80 ± 11	4.5 ± 0.86

[a] Determined for IN activity. [b] Antiviral activity.

comes. These developments suggest that IN inhibitors will be an important part of future combination therapies.

Despite dramatic advances in chemotherapy, the rates of HIV infection continue to rise, with resistance becoming an increasingly difficult problem to address. Modern treatment, including HAART, has been successful at lowering viral load to below the limits of detection, but in such cases, viral resistance eventually appears and is ultimately followed by treatment failure. The story is even more bleak for the developing world, where therapy is often not available. These points help to illustrate the continuing need for the development of new drugs, but perhaps more importantly for the developing world, the need for preventatives and vaccines.

Keywords: antiviral agents · drug design · medicinal chemistry · nucleosides · viruses

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