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Tuberculosis (2007) 87, S31–S34

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REVIEW

Development of therapeutics for AIDS: Structure-based molecular targeting

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KEYWORDS
Chemotherapeutics for HIV/AIDS;
Antiretroviral therapy;
CCR5 inhibitors;
Darunavir

Summary
Novel anti-HIV agents that target different stages of the human immunodeficiency virus type 1 (HIV-1) replication cycle are now in clinical trials and have signs of improving our ability to manage HIV-1. This chapter, and the Conference presentation, dealt with the challenges encountered in realizing the optimal benefits of the newer therapeutics to individuals with AIDS and HIV-1 infection receiving highly active antiretroviral therapy (HAART). Emphasis was on the G-protein-coupled seven-transmembrane chemokine receptor, CCR5, as a potentially new therapeutic target and experience with novel CCR5 inhibitors, and the promise from further advancement through structural and molecular analysis of CCR5 inhibitor–CCR5 interactions. The promise in some of the newer protease inhibitors was also discussed.
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Contents

Impacts of highly active antiretroviral therapy (HAART) S31
CCR5 inhibitors S32
Structural and molecular analysis of CCR5 inhibitor–CCR5 interactions S32
Darunavir, a newly FDA-approved protease inhibitor S33
More progress expected in the road map for future AIDS therapy S33
References S33

Impacts of highly active antiretroviral therapy (HAART)

HAART refers to the utilization of multiple antiviral drugs combined for treatment of human immunodeficiency virus type 1 (HIV-1) infection, and has been shown to significantly

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1472-9792/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.tube.2007.05.009

improve the quality of life in HIV-1-inflicted individuals.[1] When the first human pathogenic retrovirus termed HTLV-1 was discovered, virtually no attempt was made to explore antiretroviral therapy, since it was believed that once target cells were infected by cellular-genome-integrating retrovirus, drugs would do nothing to the progress of the retrovirus-associated diseases. The development of the first three dideoxynucleoside reverse transcriptase inhibitors (zidovudine, didanosine, and zalcitabine) made changes.[1] After these first drugs proved to be active against HIV in patients with AIDS, a number of antiviral agents were added to our armamentarium in the fight with HIV infection.[2] However, we have encountered a number of challenges in bringing about the optimal benefits of the currently available therapeutics of AIDS and HIV-1 infection to individuals receiving HAART. They include (i) drug-related toxicities; (ii) only partial restoration of immunologic functions achieved once individuals developed AIDS; (iii) development of various cancers as a consequence of survival prolongation; (iv) flare-up of inflammation in individuals receiving HAART or immune re-construction syndrome (IRS); and (v) increased cost of antiviral therapy. In particular, HIV drug resistance has become a major obstacle in the management of HIV-1 infection. Moreover, the alarming rates of transmission of drug-resistant HIV-1 variants in primary HIV-1 infection has posed another formidable problem.[3–5] Thus, the identification of a new class of antiretroviral agents that have a unique mechanism(s) of actions, cause no or minimal adverse effects, and do not allow or delay the emergence of drug-resistant HIV-1 variants, remain as crucial therapeutic objectives.

CCR5 inhibitors

Successful antiviral drugs, in theory, exert their virus-specific effects by interacting with viral receptors, virally encoded enzymes, viral structural components, viral genes, or their transcripts without disturbing cellular metabolism or function. However, at present, no antiretroviral drugs or agents are likely to be completely specific for HIV-1 or to be devoid of toxicity or adverse effects in the therapy of AIDS, which has been a critical issue because patients with AIDS and its related diseases will have to receive antiretroviral therapy for a long period of time, perhaps for the rest of their lives. In this regard, agents that block viral entry has been of particular potential therapeutic value. After the identification of CD4 as a primary receptor for HIV-1 entry into the cells of the immune system, it soon became evident that CD4 alone was not sufficient to establish productive infection. It took another 10 years until 1996 for the G-protein-coupled seven-transmembrane chemokine receptors CXCR4 and CCR5 to be identified as coreceptors for HIV-1 entry.[6] HIV-1 infection is initiated by the attachment of the virus envelope glycoprotein, gp120, to CD4 on the target cell. Binding to CD4 triggers conformational changes in gp120, which exposes a binding site for a chemokine receptor that acts as a coreceptor.[7] Interactions with the coreceptor triggers a rearrangement of the transmembrane subunit of the envelope glycoprotein, gp41, which leads to fusion between the virus and cell membrane.[8] CCR5 is an important coreceptor for macrophage-tropic (designated as R5) HIV-1 strains that are transmitted between individuals, whereas CXCR4 is the most relevant coreceptor for the T-cell-tropic (designated as X4) isolates that emerge after several years of HIV-1 infection.[6] Furthermore, blocking the function of CCR5 might not significantly impact human health, as approximately 1% of Caucasians naturally lack CCR5 because of a protein-disrupting mutation with no apparent detectable consequences.[9] Thus, CCR5 represents a potentially new therapeutic target for the development of antiretroviral agents,[10] and extensive developmental programs were launched to identify small-molecule CCR5 inhibitors. Over the past few years, we have already seen several unique small-molecule CCR5 inhibitors in the pipeline.

In 2000, we developed a novel CCR5 inhibitor, AK602/aplaviroc (AVC), which has a high affinity for CCR5 (K_D values of 3 nM), blocks HIV-1-gp120/CCR5 binding, and exerts potent activity against a wide spectrum of R5-HIV-1 isolates, including multi-drug resistant HIV-1 strains (IC_{50} values of 0.1–0.6 nM) *in vitro*.[11] In human peripheral blood mononuclear cell-transplanted R5 HIV-1_{JR-FL}-infected, non-obese diabetic-SCID interleukin-2 receptor γ-chain-knock out (NOG) mice, in which massive and systemic HIV-1 infection occurred, AVC produced ~2 $\log_{10}$ reduction in viremia.[12] In phase IIb clinical trials, patients receiving 600 mg of AVC twice daily had a mean decrease in viral load of ~1.6 $\log_{10}$ from baseline. The phase III clinical trials of AVC involving ~2000 drug-experienced patients with AIDS were implemented in the United States in the summer of 2005, however, Grade 4 hepatotoxicity occurred in a few patients and all the trials were terminated in December 2005. Two other CCR5 inhibitors developed by US pharmaceutical firms are undergoing phase III clinical studies in the United States. One of the two CCR5 inhibitors, maraviroc, has entered the Expanded Access Program in the United States.

Structural and molecular analysis of CCR5 inhibitor–CCR5 interactions

One new area in the development of therapeutics is predictive modeling. While one tends to use modeling to get to the structures of targets and potential inhibitors, there are a number of unknown factors in it and we should maximize the chances of success. In this context, we have recently characterized the structural and molecular interactions of AVC. The data obtained with saturation binding assays and structural analyses delineated the key interactions responsible for the binding of CCR5 inhibitors with CCR5, and illustrated that their binding site is located in a predominantly lipophilic pocket in the interface of extracellular loops and within the upper transmembrane domain of CCR5 (Fig. 1A). Mutations in the CCR5 binding sites of AVC decreased gp120 binding to CCR5 and the susceptibility to HIV-1 infection, while certain mutations in transmembrane domains that also decreased gp120 binding and HIV-1 infectivity had less effect on the binding of CC-chemokines, suggesting that CCR5 inhibition targeting appropriate regions might render the inhibition highly HIV-1-specific while preserving the CC-chemokine–CCR5 interactions.[13] The data delineating residue-by-residue interactions of CCR5 with CCR5 inhibitors should not only help design more

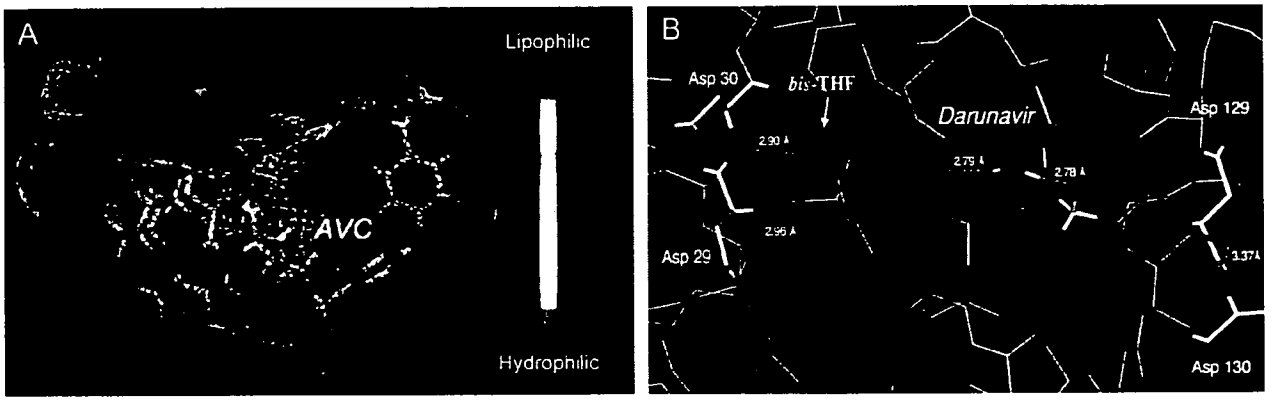

Figure 1 (A) Aplaviroc (AVC/AK602) lodged within the binding cavity of CCR5. The CCR5 cavity was defined with its lipophilic potential using MOLCAD. The region near the extracellular domain has some hydrophilic character (red arrow head), whereas the rest of the cavity is mostly lipophilic. The carboxyl and hydroxymethyl of AVC interact with the hydrophilic regions of CCR5, whereas the rest of AVC interacts with the lipophilic regions of CCR5. (B) X-ray crystal structure of HIV protease complexed with darunavir (DRV). DRV is depicted in purple and significant HIV protease residues are shown in green. Note that DRV forms tight hydrogen bond interactions with the main chains of critical active site amino acids, Asp29, and Asp30. The reason DRV exerts potent activity against various multi-drug resistant variants is presumably due to, at least in part, its interaction with the main chains (not the side chains which other PIs interact with) of these two aspartic acids.

potent and HIV-1-specific CCR5 inhibitors, but also give new insights into the dynamics of CC-chemokine–CCR5 interactions and the mechanisms of CCR5 involvement in the process of cellular entry of HIV.

Darunavir, a newly FDA-approved protease inhibitor

In other area, we have been focusing on the design and synthesis of non-peptidyl protease inhibitors (PIs) that are potent against HIV-1 variants resistant to the currently approved PIs.[14–16] One such anti-HIV-1 agent, darunavir (DRV)/TMC114, containing a structure-based designed privileged non-peptidic P2 ligand, 3(*R*),3a(*S*),6a(*R*)-bis tetrahydrofuranylurethane (bis-THF), has recently been approved as a therapeutic agent for the treatment of individuals who harbor multi-drug-resistant HIV-1 variants and do not respond to previously existing HAART regimens. DRV is extremely potent against laboratory HIV-1 strains and primary clinical isolates (IC_{50}: ~0.003 μM) with minimal cytotoxicity. DRV also blocks the infectivity and replication of HIV-1 variants exposed to and selected by various existing PIs.[15] Structurally, the close contact of DRV with the main chains (not the side chains) of the protease active site amino acids (Asp29 and Asp30) is apparently critical for the potency and wide-spectrum activity of DRV (Fig. 1B).[17]

More progress expected in the road map for future AIDS therapy

Novel anti-HIV agents that target different steps in the HIV-1 replication cycle are currently in pre-clinical trials and will undoubtedly improve our ability to manage HIV-1 infection. However, as has been the case with reverse transcriptase and protease inhibitors, the development of drug resistance will likely limit the effectiveness of new drugs as well. Thus, a key element in future drug design strategies will be to understand how drug-resistance mutations affect the interaction of the drug with its target, and to develop compounds with the adaptability to inhibit such variants along with wild-type HIV-1. New-generation reverse transcriptase and protease inhibitors have already shown promise in accomplishing this task, by utilizing knowledge of the molecular, biochemical, structural, and thermodynamic nature of drug resistance. This should serve as a model in the design of more effective anti-HIV-1 therapeutics. Moreover, the approach of combining site-directed mutagenesis-based data and molecular modeling should be useful for gaining structural insights for novel drug design. Further improved approaches to explore new treatment modalities should be continued in the hope that with new and more potent antiviral agents, we will certainly be able to control HIV-1 diseases more efficiently and effectively.

Funding: This work was supported in part by the Intramural Research Program of Center for Cancer Research, National Cancer Institute, National Institutes of Health and in part by the Grant to the Cooperative Research Project on Clinical and Epidemiological Studies of Emerging and Re-emerging Infectious Diseases (Renkei Jigyo: No. 78, Kumamoto University) of Monbu Kagakusho and Kumamoto University Research and Education Program (Kyoten) on Development of Innovative Therapy for Emerging and Re-emerging Infectious Diseases Including AIDS.

Competing interests: None declared

Ethical approval: Not required

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ACCOUNTS
of chemical research

Design of HIV Protease Inhibitors Targeting Protein Backbone: An Effective Strategy for Combating Drug Resistance

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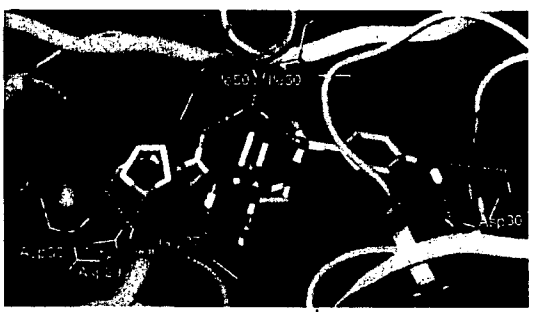
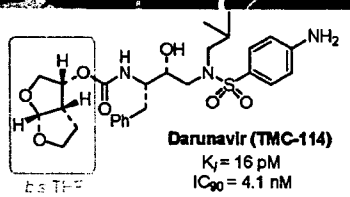
RECEIVED ON MAY 18, 2007

CONSPECTUS

The discovery of human immunodefidency virus (HIV) protease inhibitors (PIs) and their utilization in highly active antiretroviral therapy (HAART) have been a major turning point in the management of HIV/acquired immune-defidency syndrome (AIDS). However, despite the successes in disease management and the decrease of HIV/AIDS-related mortality, several drawbacks continue to hamper first-generation protease inhibitor therapies. The rapid emergence of drug resistance has become the most urgent concern because it renders current treatments ineffective and therefore compels the scientific community to continue efforts in the design of inhibitors that can efficiently combat drug resistance.





Darunavir (TMC-114)
K_i = 16 pM
IC_{90} = 4.1 nM

The present line of research focuses on the presumption that an inhibitor that can maximize interactions in the HIV-1 protease active site, particularly with the enzyme backbone atoms, will likely retain these interactions with mutant enzymes. Our structure-based design of HIV PIs specifically targeting the protein backbone has led to exceedingly potent inhibitors with superb resistance profiles.

We initially introduced new structural templates, particulary nonpeptidic conformationally constrained P_2 ligands that would efficiently mimic peptide binding in the S_2 subsite of the protease and provide enhanced bioavailability to the inhibitor. Cydic ether derived ligands appeared as privileged structural features and allowed us to obtain a series of potent PIs. Following our structure-based design approach, we developed a high-affinity 3(*R*),3a(*R*),6a(*R*)-bis-tetrahydrofuranylurethane (bis-THF) ligand that maximizes hydrogen bonding and hyrophobic interactions in the protease S_2 subsite. Combination of this ligand with a range of different isosteres led to a series of exceedingly potent inhibitors.

Darunavir, initially TMC-114, which combines the bis-THF ligand with a sulfonamide isostere, directly resulted from this line of research. This inhibitor displayed unprecedented enzyme inhibitory potency (K_i = 16 pM) and antiviral activity (IC_{90} = 4.1 nM). Most importantly, it consistently retained is potency against highly drug-resistant HIV strains. Darunavir's IC_{50} remained in the low nanomolar range against highly mutated HIV strains that displayed resistance to most available PIs.

Our detailed crystal structure analyses of darunavir-bound protease complexes dearly demonstrated extensive hydrogen bonding between the inhibitor and the protease backbone. Most strikingly, these analyses provided ample evidence of the unique contribution of the bis-THF as a P_2-ligand. With numerous hydrogen bonds, bis-THF was shown to closely and tightly bind to the backbone atoms of the S_2 subsite of the protease. Such tight interactions were consistently observed with mutant proteases and might therefore account for the unusually high resistance profile of darunavir. Optimization attempts of the backbone binding in other subsites of the enzyme, through rational modifications of the isostere or tailor made P_2 ligands, led to equally impressive inhibitors with excellent resistance profiles.

The concept of targeting the protein backbone in current structure-based drug design may offer a reliable strategy for combating drug resistance.

Published on the Web 08/28/2007 www.pubs.acs.org/acr
10.1021/ar7001232 CCC: $40.75

Introduction

Acquired immunodeficiency syndrome (AIDS), a degenerative disease of the immune system, is caused by the human immunodeficiency virus (HIV).[1,2] The current statistics for global HIV/AIDS are staggering, as an estimated 40 million people worldwide are ailing with HIV/AIDS.[3] The discovery of HIV as the etiological agent for AIDS and subsequent investigation of the molecular events critical to the HIV replication cycle led to the identification of a number of important biochemical targets for AIDS chemotherapy.[4] During viral replication, *gag* and *gag-pol* gene products are translated into precursor polyproteins. These proteins are processed by the virally encoded protease to produce structural proteins and essential viral enzymes, including protease, reverse transcriptase, and integrase.[5] Therefore, inhibition of the virally encoded HIV protease was recognized as a viable therapeutic target.[6] Since the FDA approval of the first protease inhibitor (PI) in 1995,[7] several other PIs quickly followed. The development of these PIs and their introduction into highly active antiretroviral therapy (HAART) with reverse transcriptase inhibitors marked the beginning of an important era of AIDS chemotherapy. The HAART treatment regimens arrested the progression of AIDS and significantly reduced AIDS-related deaths in the United States and other industrialized nations.[8] Despite this undeniable success, there are severe limitations of the current treatment regimens including (i) debilitating side effects and drug toxicities, (ii) higher therapeutic doses due to "peptide-like" character, and (iii) expensive synthesis and high treatment cost. Perhaps most concerning of all is the emergence of drug resistance which renders treatment ineffective in a short time. The current HAART treatment regimens are not sufficiently potent to combat multidrug-resistant HIV strains. At least 40–50% of those patients who initially achieve favorable viral suppression to undetectable levels eventually experience treatment failure.[9] Additionally, 20–40% of antiviral therapy-naive individuals infected with HIV-1 have persistent viral replication under HAART, possibly due to primary transmission of drug-resistant HIV-1 variants.[10] The development of new PIs that address this issue is essential to the future management of HIV/AIDS.

Molecular Insight and Design Strategies To Combat Drug Resistance

Our structural analysis and comparison of protein–ligand X-ray structures of wild-type and mutant HIV proteases have revealed that the active site backbone conformation of mutant proteases is only minimally distorted.[11,12] This molecular insight led us to presume that an inhibitor which makes maximum interactions in the active site of HIV protease, particularly extensive hydrogen-bonding interactions in the protein backbone of the wild-type enzyme, will also retain these key interactions in the active site of mutant proteases. Our structure-based design to combat drug resistance is guided by the premise that an inhibitor exhibiting extensive hydrogen-bonding interactions with the protein backbone of the wild-type enzyme will likely retain potency against the mutant strains, since the mutations cannot easily eliminate the backbone interactions. Our objective is then focused on designing inhibitors that specifically target and maximize these interactions with backbone atoms. Another critical issue of current HAART therapies is the poor bioavailability of the current PIs. This in turn is responsible for much of the high-dose-related severe side effects and poor compliance issues.[13] Thus, our design of ligands and templates is also focused on designing non-peptidic cyclic/heterocyclic structures with improved bioavailability. Of particular interest, we plan to design cyclic ether or polyether-derived templates as these features are common to biologically active natural products. Such polyether templates may help improving aqueous solubility and increase oral bioavailability of PIs.

Development of Bis-THF as a High-Affinity P_2 Ligand

In a preliminary investigation based upon the X-ray structure of saquinavir-bound HIV-1 protease,[14] we designed a conformationally constrained cyclic ether-derived ligand to mimic the asparagine carbonyl binding in the S_2 subsite. As shown in Figure 1, inhibitor **1** with a 3(*S*)-tetrahydrofuranylurethane displayed an enzyme IC_{50} of 132 nM. The corresponding 3(*R*)-tetrahydrofuranyl derivative was significantly less potent (enzyme IC_{50} of 694 nM).[15,16] The potency-enhancing effect of 3(*S*)-tetrahydrofuran was further demonstrated in inhibitor **2** with a hydroxylethylene isostere.[16] Subsequently, this 3(*S*)-tetrahydrofuran was incorporated in an (*R*)-(hydroxyethyl)sulfonamide isostere to provide **3** (VX-476). This low-molecular-weight protease inhibitor was later approved by the FDA as amprenavir for the treatment of AIDS.[17]

A preliminary protein–ligand X-ray crystal structure of **1**-bound HIV-1 protease indicated that the oxygen atom of the tetrahydrofuran ring may be involved in a weak interaction with the backbone NHs of Asp 29 and Asp 30.[18] In an effort to further improve the potency of inhibitor **1**, we speculated that a fused bicyclic tetrahydrofuran (bis-THF) could effectively interact with both Asp 29 and Asp 30

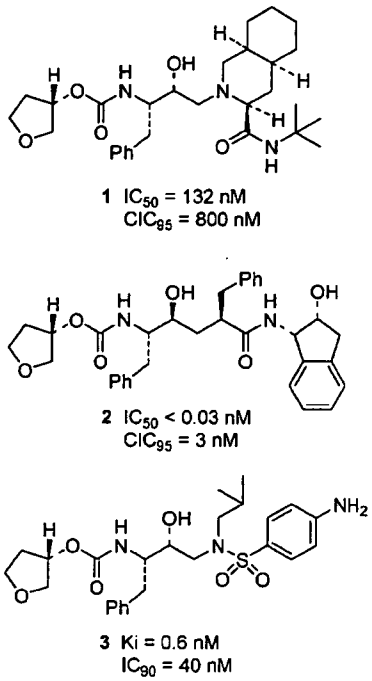


FIGURE 1. Cyclic ether-containing protease inhibitors.

amide NHs. Furthermore, the bicyclic rings of the bis-THF should offset loss of the P_3-hydrophobic quinoline ring of saquinavir. Interestingly, the bis-THF template is a subunit of ginkgolides A–C, an important class of natural products with significant biological activities.[19] Chemistry and biology of ginkgolides provided strong motivation for designing ginkgolide-derived ligands for the HIV protease substrate binding site.[19–21] Indeed, inhibitor **4** with a (3*R*,3a*S*,6a*R*)-bis-THF urethane showed a significant improvement in potency compared to **1** and its corresponding (*R*)-derivative (**5**).[15] Inhibitor **4** exhibited excellent enzyme inhibitory and antiviral potency (Figure 2).

Incorporation of the bis-THF ligand improved aqueous solubility and reduced molecular weight. Our systematic structure–activity relationship studies also ascertained that the stereochemistry (see inhibitor **5**, Figure 2), position of both oxygens (see inhibitors **6** and **7**, Figure 3), and ring sizes were critical to the activity of the inhibitor. An X-ray structure of **4**-bound HIV-1 protease revealed that both oxygens of the bis-THF ligand are within hydrogen-bonding distance to the Asp 29 and Asp 30 amide NHs in the S_2 subsite.[15]

Synthesis of the Bis-THF Ligand

The multistep synthesis of the optically active bis-THF ligand starting from (*R*)-malic acid was ineffective for the preparation of structural variants. We thus developed a three-step synthesis of racemic bis-THF followed by an immobilized lipase-catalyzed

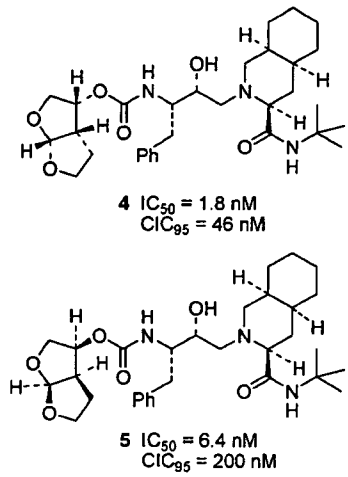


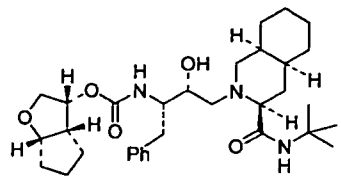
FIGURE 2. Bis-THF-containing protease inhibitors.

enzymatic resolution to provide optically active (3*R*,3a*S*,6a*R*)-3-hydroxyhexahydrofuro[2,3-*b*]furan (**12**) in high enantiomeric excess (>96% ee), as shown in Scheme 1. This synthesis helped us to extend the scope and utility of this privileged polyether-like non-peptidic ligand.[22] We recently reported two optically active syntheses of this ligand (Scheme 2). The first synthesis involved a novel stereoselective photochemical 1,3-dioxolane addition to 5(*S*)-benzyloxymethyl-2(5*H*)-furanone as the key step. The corresponding furanone was prepared in high enantiomeric excess by a lipase-catalyzed selective acylation of **15** followed by ring-closing olefin metathesis.[23] The second synthesis utilizes an ester-derived Ti–enolate-based highly stereoselective *anti*-aldol reaction as the key step.[24]

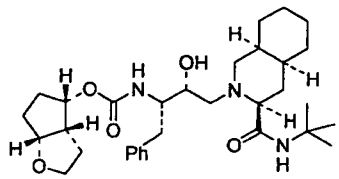
Development of Darunavir

We investigated the potency-enhancing effect of the bis-THF ligand with other isosteres. Incorporation of bis-THF in (*R*)-hydroxyethyl(sulfonamide) isosteres led to several exceedingly potent PIs with marked antiviral potency and drug-resistance profiles, as shown in Figure 4.[25]

Inhibitor **17** with a *p*-methoxysulfonamide as the P_2' ligand exhibited very impressive enzyme potency and antiviral activity. This PI has shown an excellent drug-resistance profile and good pharmacokinetic properties in laboratory animals.[26,27] It was later renamed TMC-126. In fact, inhibitor **17** showed >10-fold higher potency than the five currently available PIs (i.e., ritonavir (RTV), indinavir (IDV), saquinavir (SQV), nelfinavir (NFV), and amprenavir (APV)) in drug-sensitivity assays. It's IC_{50}s consistently remained as low as 0.3 nM.[26,27] Inhibitor **17** also displayed an unprecedented broad-spectrum activity against a large panel of primary, multidrug-resistant HIV-1 strains.[27]



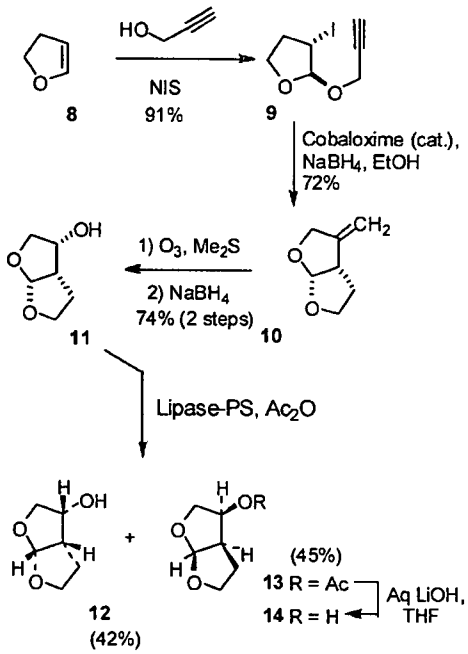
6 IC_{50} = 190 nM



7 IC_{50} = 17 nM

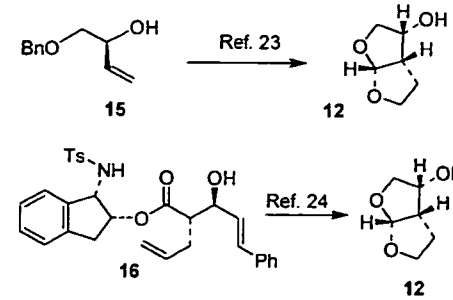
FIGURE 3. Structure of inhibitors **6** and **7**.

SCHEME 1. Efficient Optically Active Synthesis of Bis-THF Ligand



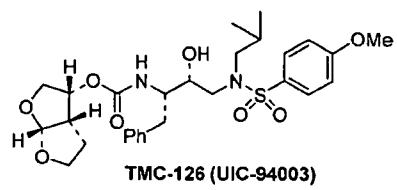
Incorporation of bis-THF into a *p*-aminosulfonamide isostere led to inhibitor **18**. Inhibitor **18** also showed unprecedented antiviral activity and outperformed most of the other currently available PIs against HIV-1_{Ba-L} by a 6–13-fold difference in IC_{50} values (Table 1).[28] Furthermore, this PI suppressed the replication of HIV-2 isolates with the most potent activity. It was later renamed TMC-114 or darunavir. When tested against HIV-1 strains that were selected for resistance to SQV, APV, IDV, NFV, or RTV after exposure to the various PIs at different concentrations (up to 5 μM), inhibitor **18** consistently and effectively suppressed viral infectivity and replication (IC_{50} values 0.003–0.029 μM) (Table 2), although lower activity was observed with APV-resistant strains (IC_{50} = 0.22 μM). In addition, inhibitor **18** potently blocked the replication of seven multidrug-resistant HIV-strains, isolated from heavily drug experienced patients with 9–14 mutations evidenced in their protease-encoding region.[28] Subsequent studies using a large panel of HIV-1 mutant strains provided further evidence of the remarkable profile of this inhibitor.[29]

SCHEME 2. Stereoselective Syntheses of the Bis-THF Ligand



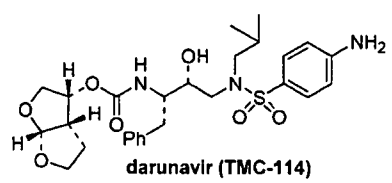
X-ray Crystal Structure of Darunavir and Evidence of Backbone Binding

High-resolution (1.10–1.34 Å) X-ray crystal structures of inhibitor **18** complexed with either wild-type HIV-1 protease or with two mutant proteases consistently showed strong hydrogen bonding of the bis-THF oxygens with the two Asp 29 and Asp 30 backbone amides (Figure 5).[28,30] New polar interactions with the Asp 30 side-chain carboxylate were also observed.[30] Additional hydrogen bonds were observed between the aniline moiety and the carbonyl oxygen and side-chain carboxylate of Asp 30′. Subsequent crystal structures of **18**-bound mutant proteases, including inhibitor **18**-bound resistant protease, clearly displayed a similar hydrogen-bonding pattern around the bis-THF ligand. These interactions seem to be crucial for maintaining the high affinity of the inhibitor



TMC-126 (UIC-94003)

17 K_i = 14 pM
IC_{90} = 1.4 nM



darunavir (TMC-114)

18 K_i = 16 pM
IC_{90} = 4.1 nM

FIGURE 4. Bis-THF-Derived Protease Inhibitors.

TABLE 1. Sensitivities of Selected Anti-HIV Agents against HIV-$1_{Ba\text{-}L}$, HIV-2_{ROD}, and HIV-2_{EHO}

		PIs, mean IC_{50} (nM) ± SDs[a]						
virus	cell type	AZT	SQV	APV	IDV	NFV	RTV	18 (TMC-114)
HIV-$1_{Ba\text{-}L}$[b]	PBMC	9 ± 1	18 ± 10	26 ± 5	25 ± 12	17 ± 4	39 ± 20	3 ± 0.3
HIV-2_{ROD}[c]	MT-2	18 ± 2	3 ± 0.2	230 ± 10	14 ± 6	19 ± 3	130 ± 60	3 ± 0.1
HIV-2_{EHO}[c]	MT-2	11 ± 2	6 ± 2	170 ± 50	11 ± 2	29 ± 18	240 ± 6	6 ± 3

[a] All assays were conducted in duplicate or triplicate; the data represent IC_{50} mean values (±SD) derived from the result of three independent experiments. [b] IC_{50} were evaluated with PHA–PBMC and the inhibition of p24 Gag protein production by the drug as an end point. [c] MT-2 cells were exposed to the virus and cultured, and IC_{50} values were determined by MTT assay.

TABLE 2. Activity of 18 against Laboratory PI-Resistant HIV-1

		IC_{50} (μM)[b]					
virus	amino acid substitution[a]	SQV	APV	IDV	NFV	RTV	18 (TMC-114)
HIV-1_{NL4-3}	wild type	0.009 (1)	0.027 (1)	0.011 (1)	0.020 (1)	0.018 (1)	0.003 ± 0.0005 (1)
HIV-$1_{SQV5\mu M}$	L10I, G48V, I54V, L90M	>1 (>111)	0.17 (6)	>1 (>91)	0.30 (15)	>1 (>56)	0.005 ± 0.0009 (2)
HIV-$1_{APV5\mu M}$	L10F, V32I, M46I, I54M, A71V, I84V	0.020 (2)	>1 (>37)	0.31 (28)	0.21 (11)	>1 (>56)	0.22 ± 0.05 (73)
HIV-$1_{IDV5\mu M}$	L10F, L24I, M46I, L63P, A71V, G73S, V82T	0.015 (2)	0.33 (12)	>1 (>91)	0.74 (37)	>1 (>56)	0.029 ± 0.0007 (10)
HIV-$1_{NFV5\mu M}$	L10F, D30N, K45I, A71V, T74S	0.031 (3)	0.093 (3)	0.28 (25)	>1 (>50)	0.09 (5)	0.003 ± 0.0002 (1)
HIV-$1_{RTV5\mu M}$	M46I, V82F, I84V	0.013 (1)	0.61 (23)	0.31 (28)	0.24 (12)	>1 (>56)	0.025 ± 0.006 (8)

[a] In PR. [b] MT-4 cells (10^4) were exposed to each HIV-1 (100x$TCID_{50}$s), and the inhibition of p24 Gag protein production by the drug was used as an end point. Numbers in parentheses represent the fold changes of IC_{50}s for each isolate relative to that of HIV-1_{NL4-3}.

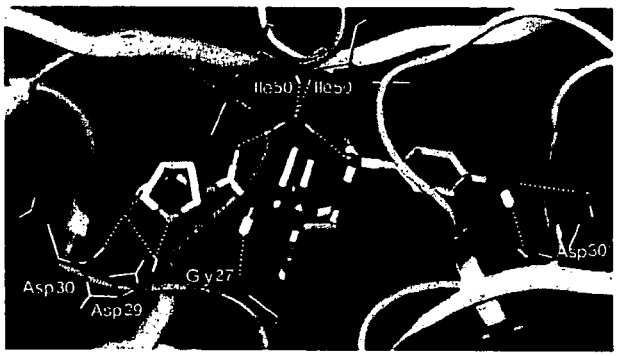


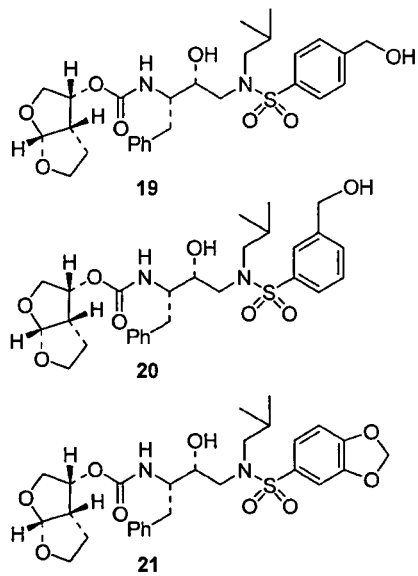
FIGURE 5. Interactions in X-ray crystal structure of **18**-bound HIV protease.

for the protease and appear to provide an explanation for the high potency against mutant proteases.[31–33]

Clinical Development of Darunavir

Inhibitor **18**, later renamed darunavir, showed a favorable pharmacokinetic profile in laboratory animals and was subsequently selected for further clinical studies. Tibotec (Belgium) carried out clinical developments of darunavir (**18**).[34] Darunavir (DRV) showed superior pharmacokinetic properties when coadministered with low doses of ritonavir.[35] Two-phase IIB clinical trials, POWER 1 and 2, are currently being performed on treatment-experienced patients to assess the safety, tolerability, and efficacy of darunavir with low doses of ritonavir (DRV/r) for 144 weeks. Early results at 24 weeks for one trial (POWER 1) showed that 77% of the DRV/r group vs. 25% for the control PI group achieved a ≥1 $\log_{10}$ viral load reduction, 53% under DRV/r vs. 18% reached a <50 cop-

TABLE 3. Sensitivity of HIV-1_{LAI} and HIV-$1_{Ba\text{-}L}$ against New PIs



			IC_{50} (nm)		
virus	cell type	assay	**19**	**20**	**21**
HIV-1_{LAI}[a]	MT-2	MTT	5.3	28	0.22
HIV-1_{LAI}[b]	PBMC	p24	2.7	8	0.22
HIV-$1_{Ba\text{-}L}$[b]	PBMC	p24	3	9.3	0.33

[a] MT2 cells (2×10^3) were exposed to 100$TCID_{50}$ of HIV-1_{LAI} culture at various concentrations of PIs. [b] The IC_{50} values were determined by exposing the PHA-stimulated PBMC to the HIV-1 strain (50$TCID_{50}$ dose per 1×10^6 PBMC) at various concentrations of PI.

TABLE 4. Activity and Cross-Resistance Profile of Inhibitor 21

virus[a]	EC_{50} (nM)					
	SQV	RTV	NFV	APV	DRV	21 (GRL-98065)
HIV-$1_{ERS104pre}$ (wild-type X_4)	8 ± 3	25 ± 5	15 ± 4	29 ± 5	3.8 ± 0.7	0.5 ± 0.2
HIV-$1_{MDR/TM\ (X4)}$	180 ± 50 (23)	>1000 (>40)	>1000 (>67)	300 ± 40 (10)	4.3 ± 0.7 (1)	3.2 ± 0.6 (6)
HIV-$1_{MDR/MM\ (R5)}$	140 ± 40 (18)	>1000 (>40)	>1000 (>67)	480 ± 90 (17)	16 ± 7 (4)	3.8 ± 0.6 (8)
HIV-$1_{MDR/JSL\ (R5)}$	290 ± 50 (36)	>1000 (>40)	>1000 (>67)	430 ± 50 (15)	27 ± 9 (7)	6 ± 2 (12)
HIV-$1_{MDR/B\ (X4)}$	270 ± 60 (34)	>1000 (>40)	>1000 (>67)	360 ± 90 (12)	40 ± 10 (11)	3.9 ± 0.5 (8)
HIV-$1_{MDR/C\ (X4)}$	35 ± 4 (4)	>1000 (>40)	420 ± 60 (28)	250 ± 50 (9)	9 ± 5 (2)	2.7 ± 0.3 (5)
HIV-$1_{MDR/G\ (X4)}$	33 ± 5 (4)	>1000 (>40)	370 ± 50 (25)	320 ± 20 (11)	7 ± 5 (2)	3.4 ± 0.3 (7)

[a] The amino acid substitutions identified in the protease-encoding region compared to the consensus type B sequence cited from the Los Alamos database include L63P in HIV-1ERS104pre; L10I, K14R, R41K, M46L, I54V,L63P, A71V, V82A, L90M, I93L in HIV-1MDR/TM; L10I, K43T, M46L, I54V, L63P, A71V, V82A, L90M, and Q92K in HIV-1 MDR/MM; L10I, L24I, I33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, and V82A in HIV-1 MDR/JSL; L10I, K14R, L33I, M36I, M46I, F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90M, and I93L in HIV-1 MDR/B; L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70Q, V82A, and L89 M in HIV-1 MDR/C; and L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, and L90 M in HIV-1 MDR/G. HIV-1ERS104 preserved as a source of wild-type HIV-1.

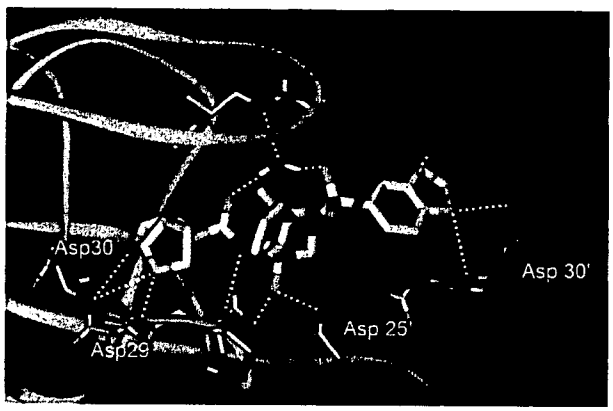


FIGURE 6. Crystal structure of inhibitor **21**-bound HIV-1 protease.

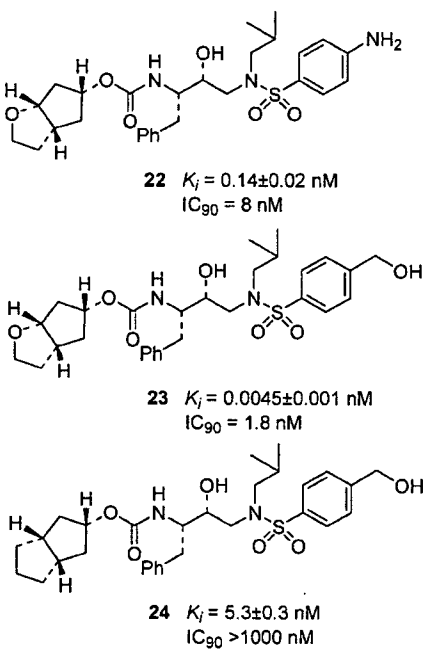


FIGURE 7. Structures of inhibitors **22–24**.

ies/mL viral load, CD_4+ cell count increased from baseline by 124 cells/mL in the DRV/r arm vs. 20 cells in the others.[36] A recent report at week 48 for the two trials showed that 61%

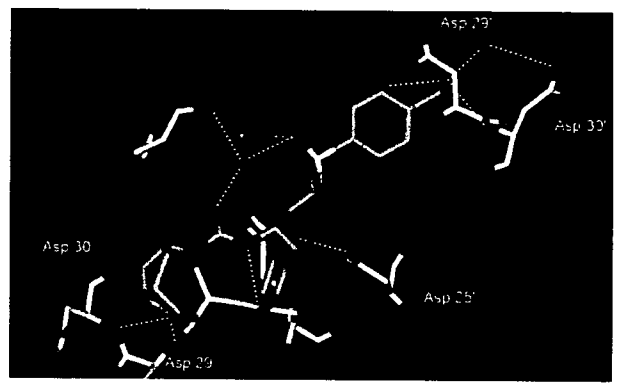


FIGURE 8. Inhibitor **23**-bound X-ray structure of HIV-1 protease.

of patients under DRV/r (600mg/100mg twice daily) maintained a >1 $\log_{10}$ reduction of viral load vs. baseline compared to 15% with the control PI arms.[37] Most impressively, 45% presented <50 viral copies/mL as opposed to 10% for the control arm. Darunavir was approved by the FDA in June 2006, as the first treatment for drug-resistant HIV.[38]

Bis-THF-Derived Novel PIs

We have further explored a number of P_2' sulfonamide functionalities to interact with the backbone atoms in the S_2' subsite. As shown in Table 3, inhibitors **19–21** displayed exceedingly potent inhibitory properties. Inhibitor **21**, which contains a benzodioxolanesulfonamide derivative as its P_2' ligand, provided impressive enzyme inhibitory (<5 pM) and antiviral potency.[39] The antiviral activity of the inhibitors was evaluated against wild-type clinical isolates HIV-1_{LAI} and HIV-1_{Ba-L} in PBMC cells and HIV-1_{LAI}-exposed MT-2 cells. Results of drug sensitivities are summarized in Table 3. Inhibitor **21**(GRL-98065) was then evaluated against both wild-type and HIV-1 mutant strains.[39] As shown in Table 4, inhibitor **21** outperformed most of the currently available PIs against multidrug-resistant HIV-1 clinical isolates, including DRV by a 2 to 10-fold improvement of activity.[39] Additional studies on

TABLE 5. Activity of 23 against a Wide Spectrum of HIV-1 Mutant Isolates

virus	mutations[a]	IC_{50} (nM) values						
		SQV	RTV	IDV	NFV	APV	DRV	**23**
1 (ET)	L10I	17	15	30	32	23	nd	3
2 (B)	L10I, K14R, L33I, M36I, M46I, F53L, K55R, I62V, L63P, A71V, G73S, V82A, L90M, I93L	230	>1000	>1000	>1000	290	10.2	15
3 (C)	I10L, I15V, K20R, M36I, M46L, I54V, K55R, I62V, L63P, K70Q, V82A, L89M	100	>1000	500	310	300	3.5	5
4 (G)	L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, L90M	59	>1000	500	170	310	3.7	20
5 (TM)	L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M, I93L	250	>1000	>1000	>1000	220	3.5	4
6 (EV)	L10V, K20R, L33F, M36I, M46I, I50V, I54V, D60E, L63P, A71V, V82A, L90M	>1000	>1000	>1000	>1000	>1000	n.d.	52
7 (ES)	L10I, M46L, K55R, I62V, L63P, I72L, G73C, V77I, I84V, L90M	>1000	>1000	>1000	>1000	>1000	n.d.	31
8 (K)	L10F, D30N, K45I, A71V, T74S	20	57	260	>1000	68	3	3

[a] Amino acids substitutions identified in the protease-encoding region of HIV-1_{ET} (ET), HIV-1_B (B), HIV-1_C (C), HIV-1_G (G), HIV-1_{TM} (TM), HIV-1_{EV} (EV), HIV-1_{ES} (ES), HIV-1_K (NFV_R) as compared to consensus B sequence cited from the Los Alamos database.

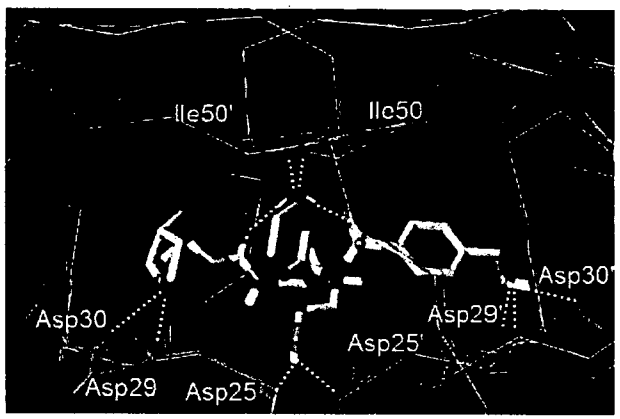


FIGURE 9. Inhibitor **23**-bound to the active site of wild-type HIV-1 protease superimposed upon the three most highly mutated drug-resistant proteases.

PI-resistant HIV-1 viral strains showed little sign of cross-resistance with inhibitor **21.**

As shown in Figure 6, the protein–ligand X-ray crystal structure of **21** revealed a pattern of four hydrogen-bonding interactions with the backbone residues of the protease similar to darunavir.[39] Because of its intriguing potency-enhancing effect and also its ability to maintain high potency against multidrug-resistant viral strains, the bis-THF ligand has been utilized for the development of other potent PIs. Most notably, researchers at GlaxoSmithKline explored an extremely potent inhibitor named brecanavir, which was a structural variant of inhibitor **21**.[40] The clinical development of this inhibitor was later abandoned reportedly due to difficulties in its formulation.

Design of Hexahydrocyclopentanofuranyl Ligand Based upon the "Backbone Binding" Concept

The remarkable ability of bis-THF-derived PIs to combat drug resistance has been documented through the clinical development of darunavir. Numerous protein–ligand X-ray crystal structures of bis-THF-containing PIs have now provided ample evidence of our concept that inhibitors with strong hydrogen-bonding interactions with the backbone atoms in the protease active site will be likely to maintain these interactions with mutant proteases and effectively combat drug resistance.[30–32] We next sought to design and develop PIs containing other novel ligands that could extensively interact with the backbone atoms. As outlined in Figure 7, we designed inhibitors **22** and **23** that contain a stereochemically defined bicyclic hexahydrocyclopentanofuran as a P_2 ligand.[41]

As shown, inhibitor **22**, with a 4-aminophenylsulfonamide as the P_2' ligand, exhibited very good enzyme inhibitory and antiviral activity. We then introduced a hydroxymethylphenylsulfonamide as a P_2' sulfonamide moiety with the intention of promoting hydrogen bonds between the hydroxyl oxygen and suitable backbone atoms in the S_2' subsite. Inhibitor **23** with a P_2' hydroxymethylphenylsulfonamide provided an impressive K_i value of 4.5 pM and antiviral IC_{50} of 1.8 nM. Compound **24** exhibited a >1100-fold loss of activity compared to that of inhibitor **23**, indicating the importance of the cyclopentanofuranyl oxygen's critical interactions in the active site. The X-ray crystal structure of **23**-bound HIV-1 protease (Figure 8) reveals that the P_2 ligand oxygen forms hydrogen bonding with the Asp 29 backbone NH.[41] The hydroxymethyl group of the P_2' sulfonamide moiety is within hydrogen-bonding distance to the Asp 30' NH as well as the side-chain carboxylate (through a 10–20° rotation of the αC–βC bond of the residue).

Inhibitor **23** has shown very impressive antiviral activity against a panel of multidrug-resistant HIV-1 variants, and the results are shown in Table 5. It exerted high potency against six other variants with IC_{50} values ranging from 4 to 52 nM.[41] All the currently available protease inhibitors tested were

highly resistant to clinical strains. Overall, inhibitor **23** is highly active against a wide spectrum of drug-resistant variants and its activity is comparable to that of darunavir.

We have compared the X-ray structure of **23** with several reported protein–ligand X-ray structures of mutant proteases. A least-squares fit of the protease α-carbons atoms was performed, allowing comparison of the interactions of **23** with each of the mutant proteases. Figure 9 depicts the superimposition of the X-ray structure of **23** with the three most highly mutated drug-resistant proteases (PDB code and color: 2F81[41] with wild type, red; 2FDD,[42] blue; 1SGU,[43] green; 1HSH,[44] yellow). As can be seen, despite multiple mutations, there is only small change in active site backbone positions. Both the P_2 ligand oxygen and the P_2' hydroxymethyl group are within hydrogen-bonding distance to the respective backbone atoms and side-chain residues in the enzyme active site. On the basis of this analysis, it appeared that inhibitor **23** should retain good to excellent contacts with the backbone of mutant proteases.

Conclusion

The emergence of drug resistance to current antiretroviral treatment represents a major challenge that needs to be addressed with the development of a new generation of inhibitors with improved pharmacological profiles. Our structure-based design of new generation protease inhibitors incorporating novel cyclic-ether-derived ligands provided exceedingly potent inhibitors with impressive drug-resistance profiles. The inhibitors are designed to make extensive interactions, particularly hydrogen bonding, with the protein backbone of HIV-1 protease. Our extensive structural analysis of protein–ligand X-ray structures of bis-THF-containing inhibitors with wild-type and mutant proteases revealed retention of strong hydrogen-bonding interactions with the protein backbone. This structural element is only slightly distorted despite multiple amino acid mutations in the active site of HIV protease. One of our designed inhibitors, darunavir, has shown superior activity against multi-PI-resistant variants compared to other FDA-approved inhibitors. It has been recently approved as the first treatment of drug-resistant HIV. This important design concept targeting the active site protein backbone may serve as an effective strategy to combat drug resistance.

Financial support by the National Institutes of Health (GM 53386, AKG) is gratefully acknowledged. This work was also supported in part by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health, and in part by a Grant-in-aid for Scientific Research (Priority Areas) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Monbu Kagakusho) and a Grant for Promotion of AIDS Research from the Ministry of Health, Welfare, and Labor of Japan. We also thank Dr. Geoff Bilcerand and Mr. Xiaoming Xu for helpful suggestions.

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FOOTNOTES

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Development of Protease Inhibitors and the Fight with Drug-Resistant HIV-1 Variants

I. Chapter Overview

The development of antiretroviral therapy for acquired immunodeficiency syndrome (AIDS) has witnessed one of the most dramatic progressions in the history of medicine. By the late 1980s, it had become apparent that combination chemotherapy with two nucleoside reverse transcriptase inhibitors (NRTIs) was more effective than NRTI monotherapy. However, only with the advent of protease inhibitors (PIs) in early 1990s, providing highly active antiretroviral therapy (HAART), significant clinical benefits became to be seen.

Advances in Pharmacology, Volume 56

1054-3589/08 $35.00
DOI: 10.1016/S1054-3589(07)56006-0

In this chapter, we discuss the principle and utility of development of PIs and the present challenges in the fight with emergence of PI-resistant HIV-1 variants.

II. Introduction

One can say that the development of antiretroviral therapy for AIDS has traced one of the most dramatic progressions in the history of medicine, showing combinations of rapid drug development, short-lived trends, and continuous evolution. In the latter half of 1980s, in the United States, efforts had been made to bring synergism to the basic research programs of the US government, private sectors, and academics on *Human immunodeficiency virus 1* (HIV-1), and to translate the basic findings into rapid development of novel therapeutics for AIDS. A focus of research on HIV-1 protease, the virally encoded enzyme has been targeted following the therapeutic success achieved by targeting at HIV-1 reverse transcriptase (Mitsuya and Erickson, 1999). Initially such an entirely new area of research was not financially well supported by industries. Moreover, the clinical utility of PIs, which had been designed using the knowledge of the molecular structure of protease, was not known. However, it had become apparent that combination chemotherapy with two NRTIs, zidovudine (azidothymidine, AZT) (Mitsuya *et al.*, 1985) and didanosine (dideoxyinosine, ddI) (Mitsuya and Broder, 1986; Yarchoan *et al.*, 1989a,b) was more effective than monotherapy as opposed to using the drugs sequentially (Yarchoan *et al.*, 1994). Between December 1995 and March 1996, three PIs, saquinavir (SQV), followed by ritonavir (RTV), and indinavir (IDV), were approved as prescription drugs for therapy of AIDS through the fast track approval mechanism by the US Food and Drug Administration (FDA) (Mitsuya and Erickson, 1999). Combination chemotherapy, with one of the PIs added to the combined NRTIs, produced sensational results in comparison to the clinical data that had been previously reported.

III. Targeting Viral Protease

A. Mechanism of Action of PIs

HIV-1 encodes a protease, also known as a proteolytic enzyme, which is responsible for the posttranslational processing of the viral products and is required for viral infectivity. Indeed, a mutation of the protease active site aspartic acids or chemical inhibition of the enzyme leads to the production of immature, noninfectious viral particles (Ghosh *et al.*, 2006a,b; Mitsuya and Erickson, 1999; Turk, 2006). The HIV-1 protease is an aspartyl protease that cleaves the HIV Gag and Gag-Pol polyproteins to generate structural

proteins and enzymes of the virus. This processing occurs late in the HIV life cycle during assembly and release from the infected cell and is an essential step for the formation of mature virus particles.

The dimeric HIV-1 protease consists of two identical monomer subunits of 99 amino acids and has an active site that lies at the dimer interface with each monomer contributing a single catalytic aspartic acid residue (Asp-25 and Asp-25′) (Fig. 1). The active site of the enzyme is unusual in that it is formed at the dimer interface and contains two conserved catalytic aspartic acid residues, one from each monomer. The substrate-binding cleft that surrounds the active site contains both hydrophobic and hydrophilic elements. Each monomer of the protease has a β-hairpin region (residues 45–56; Fig. 1) that overlaps to form a "flap" that extends over the binding cleft for the substrate. The flap is flexible enough to allow entry and exit of the polypeptide substrates and undergoes large localized conformational changes during the binding and release of inhibitors and substrates. Indeed, Hornak and their colleagues have shown using molecular dynamics simulation techniques that the unliganded HIV-1 protease flaps spontaneously open and reclose and that the flaps of the unliganded protease open to a much greater degree than observed in crystal structures and subsequently

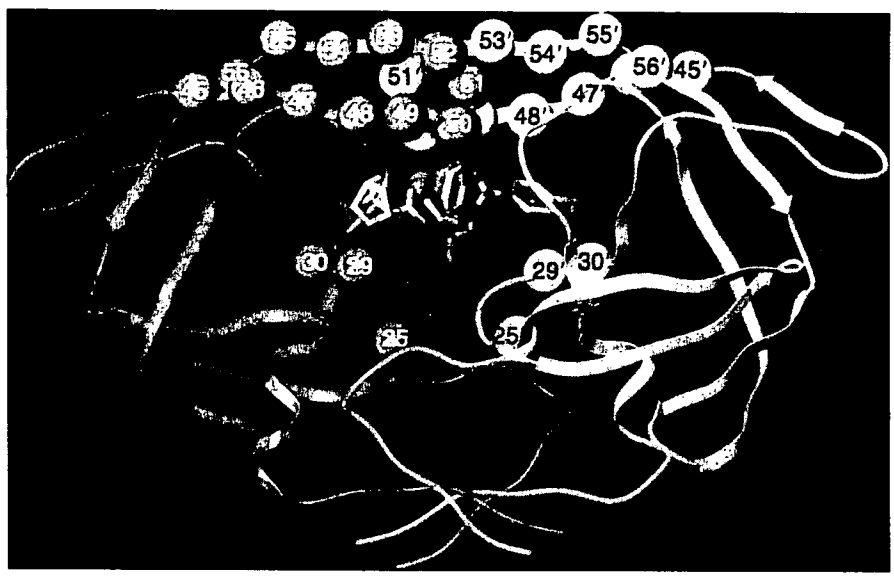

FIGURE 1 Structure of HIV-1 protease. The HIV protease consists of two identical 99 amino acid subunits and has an active site that lies at the dimer interface with each monomer contributing a single catalytic aspartic acid residue (Asp-25 and Asp-25′). Each monomer contributes amino acids (positions 45–56) to form a flap that extends over the substrate-binding cleft. The active site is covered by two β-hairpin structures or "flaps" that are highly flexible and undergo large localized conformational changes during the binding and release of inhibitors and substrates.

return to the semi-open state (Hornak *et al.*, 2006). For each substrate, three to four amino acids located on either side of the peptide bond cleavage site are utilized for binding to the substrate cavity of protease. Protease must cleave the immature HIV-1 polyprotein precursors, Gag and Gag-Pol, in at least nine different cleavage sites for maturation to occur (Jacobsen *et al.*, 1992). There is very little homology in the primary amino acid sequences of each of these cleavage sites. Instead, substrate specificity appears to be dictated by the secondary structure that remains conserved in each of the different cleavage sites.

Knowledge of the structure and functions of viral protease has led to the successful development of a wide variety of potent and chemically diverse inhibitors that have been designed using substrate- and structure-based approaches. The first PIs were designed in the early 1990s; those inhibitors were designed in such a way that the inhibitors fit exactly into the active site of the enzyme (Kempf *et al.*, 1990; Sommadossi, 1999). There are currently nine PIs approved for the treatment of HIV-1 infection (Fig. 2). All are competitive inhibitors that bind to the protease active site.

B. Protease Structures and Substrate-Based Inhibitors

In theory, antiviral drugs exert their effects by interacting with viral structural components, virally encoded enzymes, viral genomes, or specific host proteins such as cellular receptors, enzymes, or other factors required for viral replication (Mitsuya and Broder, 1987; Mitsuya and Erickson, 1999; Mitsuya *et al.*, 1990; Turk, 2006). In principle, any virus-specific steps in the replicative cycle of HIV-1, which differs from that in normal host cell function, can serve as a potential target for the development of antiretroviral therapy.

The close structural and functional relationships between retroviral and cellular aspartic proteases, together with knowledge of the HIV-1 protease cleavage site sequences on polyproteins, immediately opened an avenue of peptidomimetic substrate-based approaches that had been developed for designing inhibitors of human renin, an aspartic protease that has long been an important target for the design of antihypertensive agents. Substrate-based inhibitors are essentially peptide substrate analogues in which the scissile peptide bond has been replaced by a non-cleavable, transition-state analogue or isostere. Examples of this class of inhibitors include the first FDA-approved PI, SQV (Fig. 2), which essentially mimics the Phe-Pro cleavage site sequence (Roberts *et al.*, 1990).

C. Design of Symmetry-Based Inhibitors

With the understanding that HIV-1 protease is a twofold (C2) symmetric homodimer in which the active site is formed at the dimer interface and is

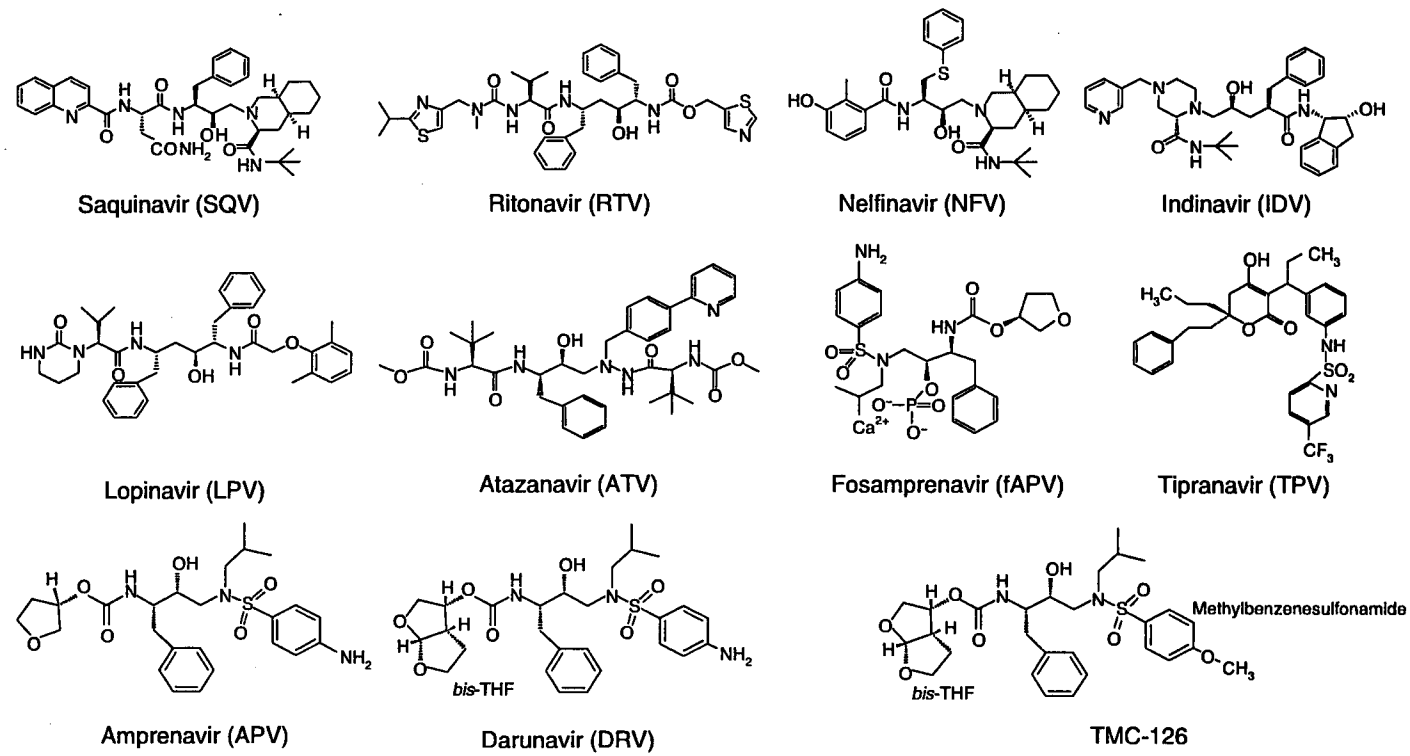

FIGURE 2 Clinically approved PIs. Structures of 10 PIs, thus far FDA-approved, are shown. Fosamprenavir is the prodrug for amprenavir. TMC-126 (not used in humans) is a prototype to darunavir.

composed of equivalent contributions of residues from each subunit came the realization that symmetry could be incorporated into the design of inhibitors for the HIV enzyme. Such designs represented a departure from traditional medicinal chemistry approaches to enzyme inhibitor designs (Erickson *et al.*, 1990; Kempf *et al.*, 1990). Examples for this type of PIs include RTV (Fig. 2).

D. Structure-Based PIs

As of today, well over 200 crystal structures have been solved and deposited in the Protein Data Bank (PDB) for various HIV-1 protease/inhibitor complexes—a testimony to the importance placed on structural information in the process of inhibitor design (Fitzgerald and Springer, 1991; Mitsuya and Erickson, 1999). Combined with medicinal chemistry and, in some cases, target-based screening efforts, these structural investigations have led to a structurally diverse compendium of inhibitors that include inhibitors like nelfinavir (NFV), that were derived solely using structure-based design methods, indinavir (IDV), and amprenavir (APV), the design of which was a blend of medicinal chemistry and structural insights (Fig. 2).

IV. The Role of PIs and Challenges in HAART

HAART, which typically exploits two reverse transcriptase inhibitors (RTIs) and one PI combined (or "boosted," *vide infra*) with RTV, has had a major impact on the AIDS epidemic in industrially advanced nations. However, no eradication of HIV-1 appears to be currently possible, in part, due to the viral reservoirs remaining in blood and infected tissues. Moreover, we have encountered a number of challenges in bringing about the optimal benefits of the currently available therapeutics of AIDS and HIV-1 infection to individuals receiving HAART (De Clercq, 2002; Siliciano *et al.*, 2004; Simon and Ho, 2003). They include (1) drug-related toxicities, (2) partial restoration of immunologic functions once individuals developed AIDS, (3) development of various cancers as a consequence of survival prolongation, (4) flame-up of inflammation in individuals receiving HAART or immune reconstruction syndrome (IRS), and (5) increased cost of antiviral therapy (Carr, 2003; Fumero and Podzamczer, 2003; Grabar *et al.*, 2006; Hirsch *et al.*, 2004; Little *et al.*, 2002).

Unlike the case for the majority of RTIs, most HIV-1 PIs had pharmacokinetic limitations. Poor oral absorption, serum–protein binding, and liver enzyme metabolism can eliminate the antiviral benefits of many otherwise highly potent PIs. PIs need to be ingested often and in large quantities to maintain effective antiviral concentrations in the blood. Furthermore, of the currently available antiviral drugs for HIV-1 infection, PIs are among the most effective, but they are costly and require complicated treatment regimens. Problematic