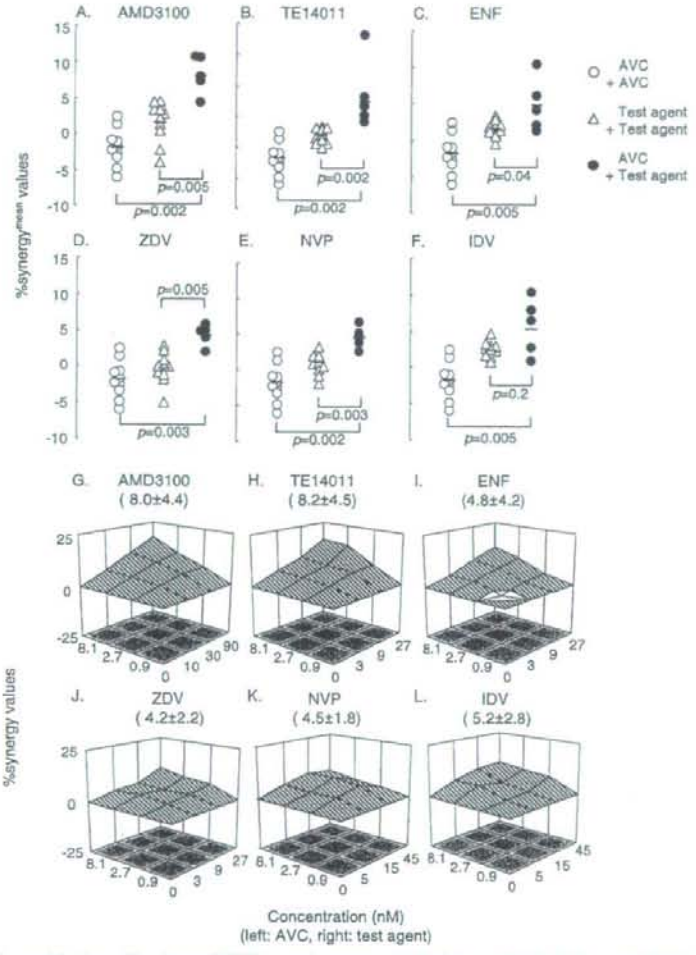

FIG. 5. Effects of AVC in combination with other anti-HIV-1 agents against a 50:50 mixture of R5-HIV-1_{Ba-L} and X4-HIV-$1_{ERS104pre}$. PHA-PBMCs were exposed to a 50:50 mixture of R5-HIV-1_{Ba-L} and X4-HIV-$1_{ERS104pre}$ and cultured in the presence of AVC in combination with AMD3100 (A), TE14011 (B), ENF (C), ZDV (D), NVP (E), or IDV (F) for 7 days, and the amounts of p24 Gag proteins in the culture supernatants were determined. Differences in the %synergy^{mean} values between AVC-AVC, test agent-test agent, and AVC-test agent were examined by using the Wilcoxon rank sum test. A statistically significant difference, or a strong trend, was observed for all combinations except between IDV-IDV and AVC-IDV ($P = 0.2$). The short bars indicate the arithmetic means obtained. The average percent synergy values for the AVC-test agent combinations were also plotted in 3-D graphs, as determined on the basis of the method of Prichard et al. (see the legend to Fig. 2) (G to L). Assays with AVC in combination with each drug and the same-drug combination were performed 5 times and 10 times, respectively. All assays were conducted in duplicate.

It was apparent that the %synergy^{mean} values of AVC-AMD3100 and AVC-TE14011 were greater than those of AVC plus any of the four antiviral agents (ENF, ZDV, NVP, and IDV). We therefore examined whether the apparent differences were statistically significant using the Wilcoxon rank sum test. The %synergy^{mean} values for AVC-AMD3100 were greater than those for AVC-ZDV ($P = 0.0472$) and AVC-NVP ($P = 0.0472$) but not those for AVC-ENF ($P = 0.1745$) and AVC-IDV ($P = 0.4647$). The %synergy^{mean} values of AVC-TE14011 were not statistically different from those of AVC plus any of the four agents ($P > 0.05$). Thus, even on the basis of a limited number of experiments, there were cases in which AVC-AMD3100 produced statistically greater synergism compared with the levels of synergism obtained with combinations of AVC and other conventional drugs. However, given the fact that the methodology used in the present study may as yet produce overestimates of synergy and the variability of the responses obtained by comparison of the combinations may also affect the analysis, it should be stressed that the effects of combinations of another CCR5 inhibitor(s) with another CXCR4 inhibitor(s) should be examined to confirm such synergism.

DISCUSSION

In the present study, we demonstrated that AVC exerts synergistic activity against HIV-1_{Ba-L} in vitro when it is combined with ZDV, NVP, IDV, or ENF. These results are generally consistent with the data reported by Tremblay and colleagues, who examined two experimental CCR5 inhibitors, TAK220 and SCH-C, in combination with ZDV, lamivudine, IDV, efavirenz, or ENF for their effects against R5-HIV-1 isolates (38, 39).

Another CCR5 inhibitor, MVC, has recently received accelerated approval by the FDA for use in combination with other antiretroviral agents for the treatment of R5-HIV-1 in adults whose viral loads remain detectable despite existing antiviral treatment or who have multiple-drug-resistant viruses. In a recent 48-week data set from the MERIT study conducted with antiretroviral therapy-naïve subjects infected with R5-HIV-1 (32), 70.6% of the patients receiving MVC achieved HIV-1 RNA levels of less than 400 copies/ml, whereas 73.1% of the patients in the efavirenz group achieved HIV-1 RNA levels of less than 400 copies/ml, which met the criteria for noninferiority. However, when the HIV-1 RNA cutoff level of less than 50 copies/ml was used, noninferiority could not be confirmed. These data suggest that some of the patients who were determined to harbor R5-HIV-1 and who failed MVC therapy may have had X4-HIV-1, which MVC does not suppress.

It may be reasonable to suggest that R5-HIV-1 is not highly dominant in those infected with HIV-1. Indeed, the prevalence levels of R5- and X4-tropic viruses in patients with HIV-1 infection vary depending on the cohort examined. Demarest et al. demonstrated that the majority of drug-naïve and drug-experienced HIV-1-infected individuals harbored R5-HIV-1 (88% and 67%, respectively), while a mixture of R5- and X4-tropic viruses was seen in 12 and 28% of those individuals, respectively, and X4-tropic virus was seen in only 0 and 5% of those individuals, respectively (8). Fätkenheuer et al. have reported that the overall prevalence of R5-HIV-1 was 94% among the individuals whom they examined (11), while Daar et al. showed that 59.5% of 126 children and adolescents harbored R5-HIV-1 and the rest (40.5%) harbored viruses with dual or mixed tropisms (5). Taken together, the presence or absence of X4-HIV-1 in individuals who are to receive a CCR5 inhibitor, regardless of their positivity for X4-HIV-1, appears to be a critical factor for successful treatment with CCR5 inhibitor-containing regimens.

Using the Combo method (see below), we found that there were significant synergistic effects when AVC was combined with ZDV, NVP, IDV, or ENF and tested against HIV-$1_{Ba-L/104pre}$ (Fig. 5C to F). Interestingly, only a lower level of synergism was observed when AVC was combined with TAK779. When AVC was combined with SCH-C, no synergism was seen (Fig. 4A and C). In this regard, we have previously observed that the binding pockets for AVC, SCH-C, and TAK779 are all located in the same hydrophobic cavity within CCR5, although their binding profiles differ substantially from each other (17). We analyzed the interactions of these three inhibitors in relation to wild-type CCR5 ($CCR5_{WT}$). When $CCR5_{WT}$-overexpressed Chinese hamster ovarian cells were exposed to ^{3}H-labeled AVC, followed by the addition of unlabeled SCH-C, ^{3}H-labeled AVC binding to CCR5 was reduced only moderately. When the interaction between ^{3}H-labeled AVC and unlabeled TAK779 was examined, ^{3}H-labeled AVC binding to $CCR5_{WT}$ was not significantly replaced by unlabeled TAK779 binding. On the contrary, when ^{3}H-labeled SCH-C was added first and then unlabeled AVC was added, the binding of ^{3}H-labeled SCH-C to $CCR5_{WT}$ was significantly blocked, suggesting that AVC effectively replaced the ^{3}H-labeled SCH-C and bound to $CCR5_{WT}$. The binding of ^{3}H-labeled TAK779 was likewise blocked by the addition of unlabeled AVC, although the extent of replacement of ^{3}H-labeled TAK779 by AVC was less compared to the extent of replacement of ^{3}H-labeled SCH-C by AVC. These results appear to be explained at least in part by the binding of these three inhibitors to the same hydrophobic cavity within CCR5, although their binding profiles are different from each other and their affinities of binding to $CCR5_{WT}$ are also different (17). Whatever the mechanism, the present data suggest that the combination of small-molecule CCR5 inhibitors does not seem to bring about synergistic activity and that caution should perhaps be used when the use of combinations of multiple CCR5 inhibitors is considered.

Notably, the most potent synergism was seen when AVC was combined with AMD3100 or TE14011, as examined against HIV-$1_{Ba-L/104pre}$ (Fig. 5A and B). The synergy values for AVC-AMD3100 and AVC-TE14011 were greater (8.0 ± 4.4 and 8.2 ± 4.5, respectively) than those for any other combination (Fig. 5G and H). Hirsch's group has also examined the effects of the combination of aminooxypentane-RANTES and a derivative of stroma-derived factor 1β, using a mixture of R5- and X4-HIV-1 isolates, and found that these two agents effectively suppressed their replication, although they did not compare the effects of CCR5 inhibitors or CXCR4 inhibitors plus other FDA-approved anti-HIV-1 agents (31). The mechanism of the potent synergism with AVC plus each of the two CXCR4 inhibitors observed in the present study is not clear at this time. In this regard, Singer et al. have demonstrated that CCR5, CXCR4, and CD4 are apposed predominantly on cellular microvilli and apparently form homogeneous microclusters in all cell types examined, including macrophages and T cells (34). Such a spatial distribution of the surface cellular molecules involved in HIV-1's cellular entry may be related to the observed antiviral synergism, possibly through the concurrent CCR5 inhibitor and CXCR4 inhibitor binding to target molecules, which might result in synergistic steric hindrance or conformational changes in such surface molecules, leading to the inhibition of HIV-1's gp120/gp41 binding to and/or fusion with the target cells.

In the present study, in order to assess the effects of the combination of AVC with other drugs, we developed a system, designated the Combo method, which provides (i) a flexible choice of interaction models, (ii) the use of nonparametric statistical methods to obtain *P* values for comparisons, and (iii) flexibility with respect to the experimental design (e.g., checkerboard and constant-ratio designs). It is of note that when AVC was combined with itself, there was an indication of synergism, as assessed by CIs greater than CI at an inhibitory effect of 75%. This result was thought to represent a limitation or error inherent to the variability of the cell-based assay data obtained and/or the median-effect method used. Indeed, as has been noted by others (21), a "combination effect" is often defined on the basis of the empirical CI values (e.g., <0.9 for synergy and >1.1 for antagonism), irrespective of the interassay variability, and no adjustments for multiple comparisons

are generally made, producing an increased potential to overestimate the combination effects.

The variability of the cell-based assay data in determining the biological profiles of HIV-1, including its infectivity, replication competence, and cytopathic effect, stems from the very nature of the replication profiles of HIV-1 in culture. Unlike the bacterial multiplication profile, for which inhibition by antibiotics is arithmetically more predictable than is the case with HIV-1 replication, HIV-1 replication involves profuse but variable numbers of infectious progeny virions produced from a single infected cell. Indeed, the estimated number of progeny HIV-1 virions produced by a single HIV-1-exposed cell in our previous study ranged from 3.5×10^2 to 1.2×10^3 (14), while Layne et al. reported that such numbers ranged from 6×10^2 to 2.6×10^6 virions per cell (15). Moreover, the infectivity and replication competence of the HIV-1 inoculum can vary in the interassay as well as the intra-assay context. In fact, it is difficult to determine what portion of the virions used in a cell-based assay is infectious and replication competent. Layne et al. estimated the ratios of infectious to noninfectious virions using syncytium-forming units, which represents the infection of individual target cells by the number of cell-free virus counted (24) and which ranges from 4.1×10^{-4} to 3.6×10^{-7} (15). However, the number of virions produced in culture and their infectious potency also vary depending on the types of cells, particularly when target PHA-PBMCs are generated from different donors. Also, there should be variability in terms of the infectivity depending on the conditions of how and where the virus inoculum was generated and stored prior to the assay (16). In addition, if 100% viral infectivity suppression is not achieved in cell culture, a viral breakthrough tends to occur, since a continuous increase in the HIV-1 inoculum size occurs over the culture period and also contributes to the variability of the antiviral data. It is also true that the cell-based assays measure the cumulative effects of inhibitors over multiple cycles, which results in a substantial overestimation of synergy (12). It is noteworthy that the standard deviations of the EC_{50}s of the three CCR5 inhibitors (AVC, SCH-C, and TAK779) were relatively large (Table 1). In this regard, we have previously reported that much greater variability in the EC_{50}s for AVC compared to that for other classes of antiretroviral agents can be seen (18, 23). This variability most likely stems from that fact that the amounts of CCR5 receptors expressed on PBMCs differ substantially from one PBMC donor to another. Thus, evaluations of the antiviral effects of drug combinations require cautious interpretation of the data, including the use of statistical analyses to judge whether the differences between drug combinations are significant.

Using the Combo method described here, we compared the %synergy^{mean} values for AVC-AMD3100 with those for AVC-ENF, AVC-ZDV, AVC-NVP, and AVC-IDV (Fig. 5). Except in two instances, the effect of the combination of AVC with AMD3100 or TE14011 did not statistically exceed the effects of AVC plus other FDA-approved antiviral agents (Fig. 5) when the %synergy^{mean} values were examined. This observation that the synergism of AVC and a CXCR4 inhibitor failed to significantly exceed the effect of AVC in combination with the other antiviral agents tested could be due to the fact that the present study was not formally designed or powered for a direct comparison of the %synergy^{mean} values of AVC-AMD3100 and AVC-TE14011 against those of AVC plus FDA-approved antiretroviral drugs, with the result being that the number of experimental results evaluated for this comparison was quite small ($n = 5$). Larger experiments would likely be able to detect these differences as being significant. However, in our limited experiments, there was a tendency for the %synergy^{mean} values to be greater when AVC was combined with CXCR4 inhibitors than when AVC was combined with other compounds, even when this was not demonstrated statistically.

In the present study, cytotoxicity was virtually negligible at all concentrations and with all combinations examined. However, it is of note that although in the preclinical testing of AVC it was administered to monkeys at high doses over 9 months and no toxicity was observed, in phase IIb clinical trials, AVC caused grade 4 hepatotoxicity in 5 of 281 individuals receiving the drug and its development was abruptly terminated (25). Nevertheless, another CCR5 inhibitor, MVC, has been well tolerated, exerts significant antiviral effects, and has now been clinically used, suggesting that the hepatotoxicity of AVC is due to its chemical/structural properties and is not inherent to CCR5 inhibitors.

In conclusion, the present data demonstrate a tendency toward greater synergism when AVC is combined with either of the two CXCR4 inhibitors than when AVC is combined with other FDA-approved anti-HIV-1 agents, suggesting that the development of effective CXCR4 inhibitors may be important to increasing the efficacies of CCR5 inhibitors.

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

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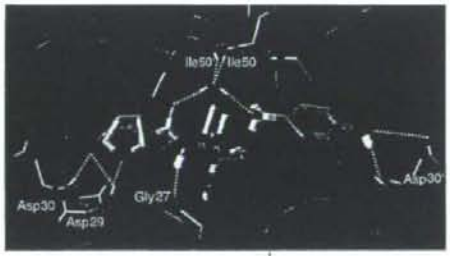
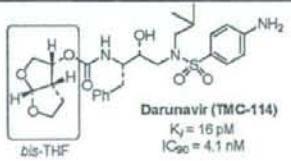
CONSPECTUS

The discovery of human immunodeficiency 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-deficiency 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. Cyclic 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 clearly 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.

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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.

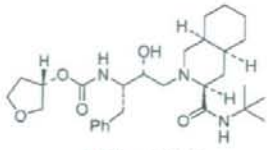
Molecular Insight and Design Strategies To Combat Drug Resistance

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.

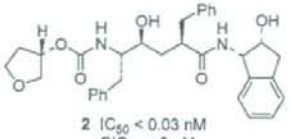
Development of Bis-THF as a High-Affinity P_2 Ligand

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]

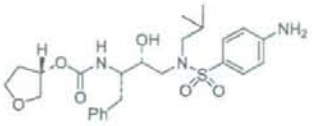
A preliminary protein–ligand X-ray crystal structure of **1**-bound HIV-1 protease indicated that the oxygen atom of the tetrahydrofuran ring may be involved in a weak interaction with the backbone NHs of Asp 29 and Asp 30.[18] In an effort to further improve the potency of inhibitor **1**, we speculated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



1 IC_{50} = 132 nM
CIC_{95} = 800 nM



2 IC_{50} < 0.03 nM
CIC_{95} = 3 nM



3 Ki = 0.6 nM
IC_{90} = 40 nM

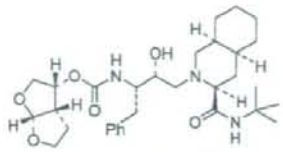
FIGURE 1. Cyclic ether-containing protease inhibitors.

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).

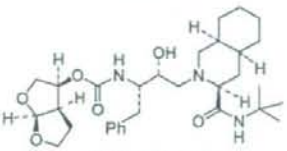
Incorporation of the bis-THF ligand improved aqueous solubility and reduced molecular weight. Our systematic structure–activity relationship studies also ascertained that the stereochemistry (see inhibitor **5**, Figure 2), position of both oxygens (see inhibitors **6** and **7**, Figure 3), and ring 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



4 IC_{50} = 1.8 nM
CIC_{95} = 46 nM



5 IC_{50} = 6.4 nM
CIC_{95} = 200 nM

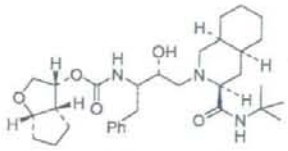
FIGURE 2. Bis-THF-containing protease inhibitors.

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]

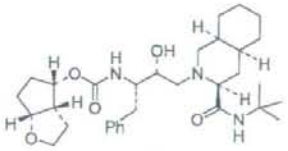
Development of Darunavir

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]



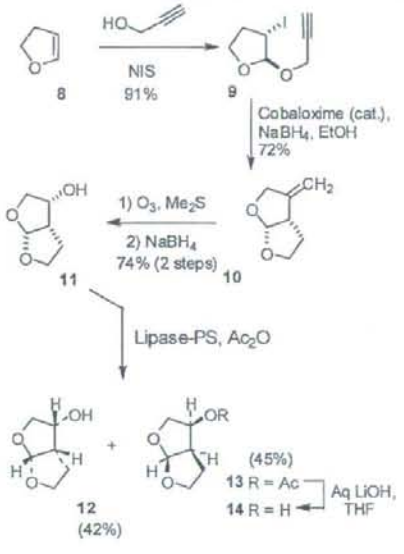
6 IC_{50} = 190 nM



7 IC_{50} = 17 nM

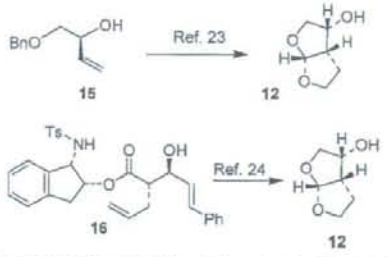
FIGURE 3. Structure of inhibitors **6** and **7**.

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



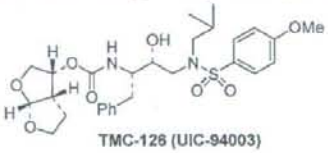
Incorporation of bis-THF into a *p*-aminosulfonamide isostere led to inhibitor **18**. Inhibitor **18** also showed unprecedented antiviral activity and outperformed most of the other currently available PIs against HIV-1_{Ba-L} by a 6–13-fold difference in IC_{50} values (Table 1).[28] Furthermore, 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



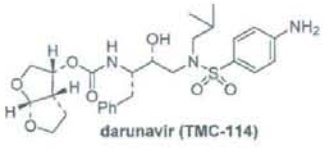
X-ray Crystal Structure of Darunavir and Evidence of Backbone Binding

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-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-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-$1_{SQVS\mu M}$	L10I, G48V, I54V, L90M	>1 (>111)	0.17 (6)	>1 (>91)	0.30 (15)	>1 (>56)	0.005 ± 0.0009 (2)
HIV-$1_{APVS\mu M}$	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-$1_{IDVS\mu M}$	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-$1_{NFVS\mu M}$	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-$1_{RTVS\mu M}$	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}.

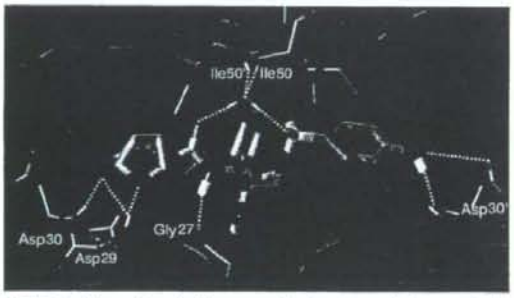


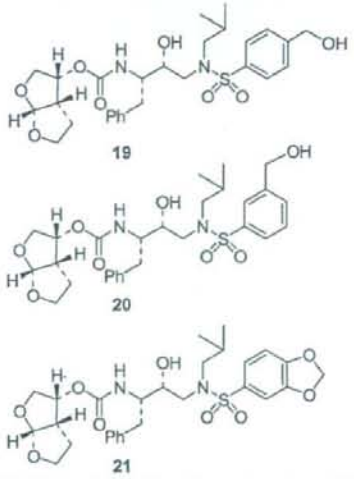
FIGURE 5. Interactions in X-ray crystal structure of **18**-bound HIV protease.

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-L} against New PIs



19

20

21

			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-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.

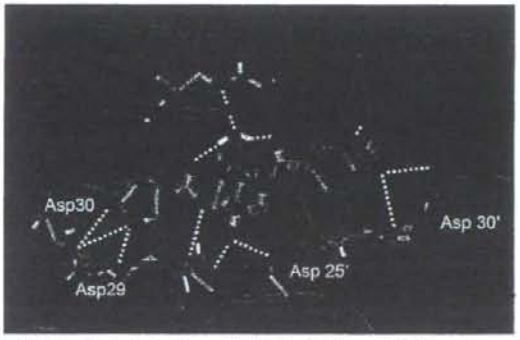


FIGURE 6. Crystal structure of inhibitor **21**-bound HIV-1 protease.

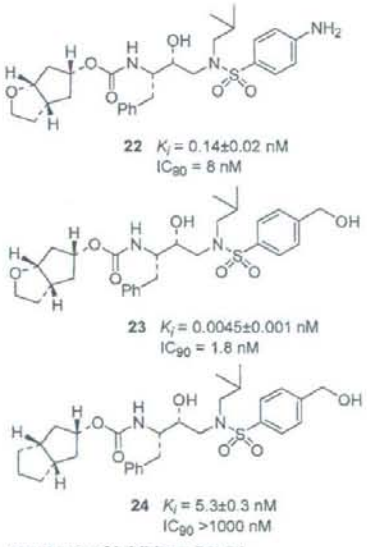


FIGURE 7. Structures of inhibitors **22–24**.

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%

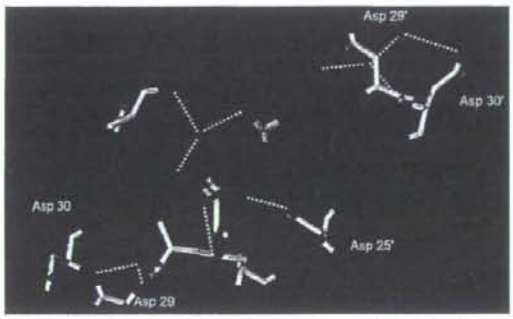


FIGURE 8. Inhibitor **23**-bound X-ray structure of HIV-1 protease.

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_X (NFV_R) as compared to consensus B sequence cited from the Los Alamos database.

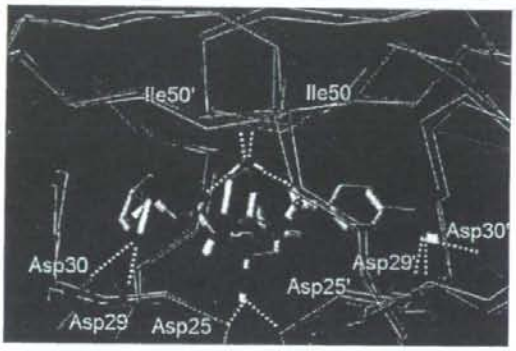


FIGURE 9. Inhibitor **23**-bound to the active site of wild-type HIV-1 protease superimposed upon the three most highly mutated drug-resistant proteases.

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.

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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.

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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

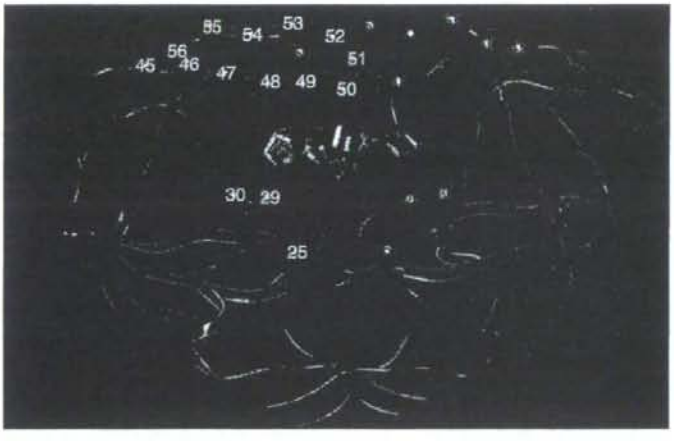

FIGURE 1 Structure of HIV-1 protease. The HIV protease consists of two identical 99 amino acid subunits and has an active site that lies at the dimer interface with each monomer contributing a single catalytic aspartic acid residue (Asp-25 and Asp-25′). Each monomer contributes amino acids (positions 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

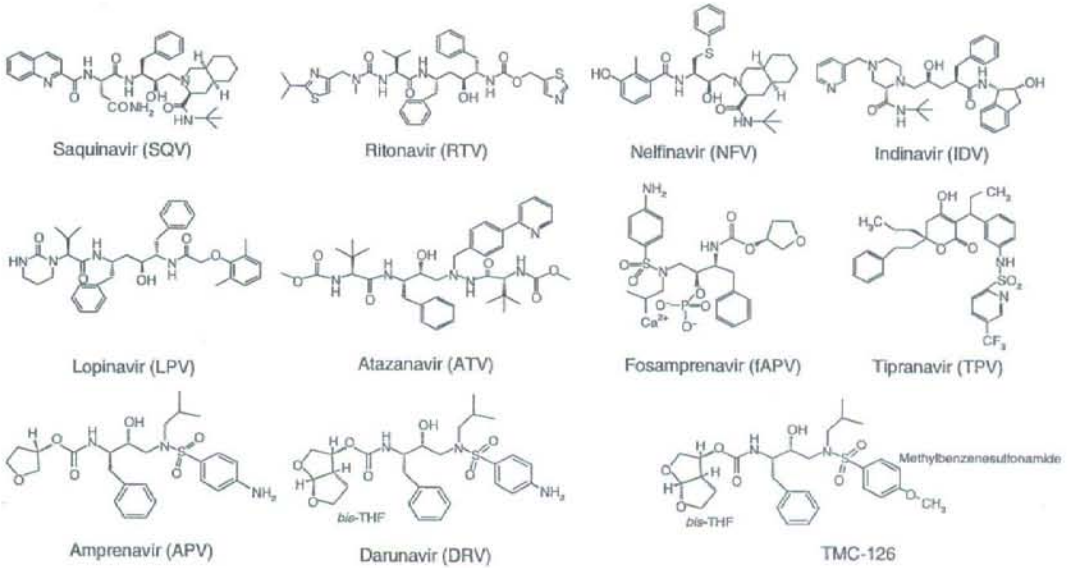

FIGURE 2 Clinically approved PIs. Structures of 10 PIs, thus far FDA-approved, are shown. Fosamprenavir is the prodrug for amprenavir. TMC-126 (not used in humans) is a prototype to darunavir.

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