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Bis-Tetrahydrofuran: a Privileged Ligand for Darunavir and a New Generation of HIV Protease Inhibitors That Combat Drug Resistance

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Introduction

The acquired immunodeficiency syndrome (AIDS) epidemic continues to be a major challenge in medicine worldwide. According to the World Health Organization (WHO), the total number of people living with the human immunodeficiency virus (HIV) has reached an estimated 40.3 million, including nearly 5 million people newly infected with the virus in 2005.[1] The magnitude of the HIV/AIDS pandemic is truly astounding. Soon after the discovery of HIV as the etiological agent for AIDS, many biochemical targets were identified to combat this devastating disease.[2,3] During viral replication, *gag* and *gag-pol* gene products are translated as precursor polyproteins. These proteins are processed by a virally encoded protease to provide structural proteins (p17, p24, p9, and p7) and essential viral enzymes (including protease, reverse transcriptase, and integrase).[4] As a consequence, the retroviral enzymes reverse transcriptase (RT), integrase (IN), and protease (PR) were identified as potential drug targets. Therapeutic inhibition of the virally encoded HIV protease became particularly attractive because of prior knowledge of mechanism-based inhibition of other aspartyl proteases. In a combination therapy with a reverse transcriptase inhibitor, the protease inhibitor saquinavir (Invirase, **1**) discovered by researchers at Hoffman–LaRoche, was the first to receive approval by the United States Food and Drug Administration (FDA) in 1996 for the treatment of AIDS.[5,6] To date, a number of other protease inhibitors have been approved, and several others are undergoing advanced clinical trials. Combination therapy or highly active antiretroviral therapy (HAART), which uses HIV protease inhibitors and reverse transcriptase inhibitors, has become the major treatment regimen for AIDS.[7] Whereas HAART therapies have definitely improved the course of HIV management and halted the progression of AIDS, the majority of protease inhibitors contain substantial peptide-like features. As a result, anti-protease therapy suffers from the traditional problems of peptide-based drugs such as poor absorption, aqueous solubility, and metabolic instability. The most alarming is the rapid emergence of drug resistance, rendering these therapies ineffective.[8] Conceivably, new-generation nonpeptidal protease inhibitors that maintain potency against mutant strains resistant to the currently approved protease inhibitors may substantially delay the emergence of clinical resistance and may alleviate the problems of "peptide-based" drugs.[9] Thus, ready availability of a number of protein–ligand X-ray crystal structures and many elegant structure–activity studies have provided new opportunities and challenges for the structure-based design of protease inhibitors to combat drug resistance.[10,11]

Background

Human immunodeficiency virus (HIV) is a member of the lentivirus subfamily of retroviruses and, like other retroviruses, contains three major genes (*gag*, *pol*, and *env*).[12] The *pol* gene encodes for the enzymes reverse transcriptase (RT), integrase (IN), and protease (PR), which are critical for viral replication. Viral assembly begins with the association of the genomic RNA with the *gag* and *gag-pol* polyproteins, the primary translational products of the viral genome. The function of the protease is to cleave the polyproteins into functional proteins essential for the production of infectious progeny virus. The active form of the protease is a homodimeric endopeptidase of the aspartyl protease family.[13] Each monomer is made up of 99 amino acids, each contributing an aspartic acid residue to form the catalytic site.[13] Inactivation of the protease by either site-di-

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rected mutagenesis or chemical inhibition leads to the production of immature, noninfectious viral particles, thus making the protease an attractive target for antiviral therapy.[14] Based on the transition-state mimetic concept that uses various nonhydrolyzable hydroxyethylene and hydroxyethylamine isosteres, an incredible effort has been carried out by academic and pharmaceutical research laboratories to design and develop potent protease inhibitors (PIs).[15] Early research involved the discovery of peptidomimetic inhibitors. More current emphasis has been to minimize molecular size, decrease peptide-like features, and design functionalities to combat drug resistance. Beside saquinavir (SQV, **1**),[5,6] seven other protease inhibitors have also been approved by the FDA for the treatment of AIDS in combination with reverse transcriptase inhibitors (Figure 1). These include: indinavir (IDV, **2**),[16] nelfinavir (NFV, **3**),[17] ritonavir (RTV, **4**),[18] atazanavir (ATV, **5**),[19] lopinavir (LPV, **6**),[20] amprenavir (APV, **7**),[21] and tipranavir (TPV, **8**).[22] Nelfinavir (**3**) and lopinavir (**6**) possess the same core unit as saquinavir (**1**) and ritonavir (**4**), respectively. However, the pharmacological properties and drug resistance profiles of **3** and **6** are very different from the corresponding inhibitors **1** and **4**.

In the clinical setting, all of these protease inhibitors have shown remarkable effectiveness.[23] As many as 90% of the clinical trial participants who received a protease inhibitor along with zidovudine (AZT) and lamivudine (3TC) have shown reduced viral load and increased CD_4^+ lymphocyte counts.[24] The introduction of these highly active antiretroviral therapies (HAART) arrested the progression of AIDS[25] and significantly reduced AIDS-related deaths in the United States and other industrialized nations. There is no doubt that HAART treatment regimens dramatically improved the quality of life and survival of patients infected with HIV, however, their ability to provide effective long-term antiretroviral therapy for HIV infection has become a complex issue. There are serious limitations with all of the currently approved protease inhibitors, including: 1) debilitating side effects and drug toxicities, 2) higher therapeutic doses due to "peptide-like" character, 3) expensive synthesis and high treatment cost, and most concerning, 4) the emergence of drug resistance. At least 40–50% of those patients who initially achieve favorable viral suppression to undetectable levels experience treatment failure.[26] Additionally, 20–40% of antiviral therapy-naive individuals infected with HIV-1 have persistent viral replication (plasma HIV RNA $>$ 500 copies mL^{-1}) under HAART, possibly due to transmission of drug-resistant HIV-1 variants.[27] In addition to the issue of drug resistance, tolerance and adherence to complex medical regimens are becoming critical issues. The drugs must be taken in gram quantities daily because of low oral bioavailability. Most currently approved PIs are associated with complex side effects including peripheral lipodystrophy, hyperlipidemia, and insulin resistance. Thus, current designs and syntheses of a new class of PIs are faced with the following major challenges: 1) improvement of potency and pharmacokinetic properties which can substantially reduce therapeutic doses, maximize effectiveness, and reduce side effects; 2) design of inhibitors that can effectively combat drug resistance; and 3) cost-effective synthesis of PIs to make these drugs readily accessible to third world countries, where the epidemic continues to worsen. To address various issues of PI therapy, our research emphasis has been focused on the design and synthesis of nonpeptidal protease inhibitors that are potent against mutant strains resistant to the currently approved protease inhibitors.

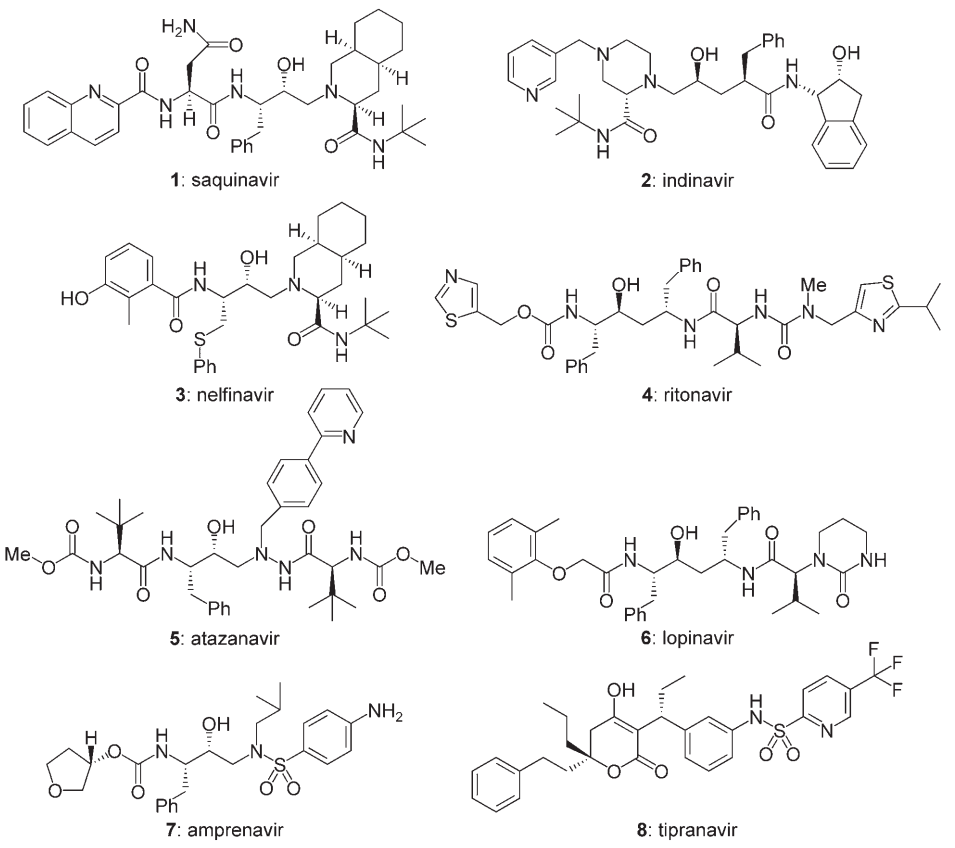
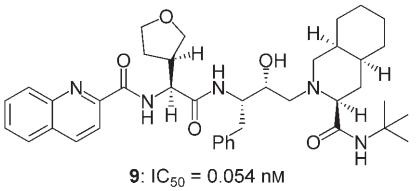


Figure 1. FDA-approved HIV protease inhibitors for the treatment of HIV infection and AIDS.

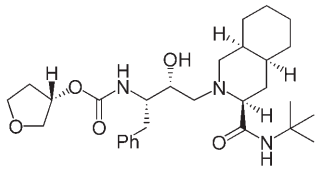
Structure-Based Design of Cyclic-Ether-Derived Nonpeptide P_2 Ligands

In an effort to reduce peptidic features, molecular weight, and structural complexity of the current protease inhibitors, we have designed a number of nonpeptidal high-affinity ligands for the HIV protease substrate binding site. The ligands are specifically designed based on various available three-dimen-

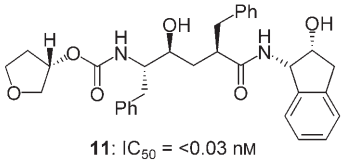
sional structures of the protein–ligand complex. One of the important elements in this design is to incorporate a stereochemically defined and conformationally constrained cyclic ether that will replace peptide bonds, mimic the biological mode of action, and make maximum interactions in the active site including hydrogen bonding with the protein backbone. The idea of incorporating cyclic ethers is from the observation that a number of naturally occurring biologically active motifs comprise cyclic ether as one of their epitopes. On the basis of this presumption, Ghosh et al. developed 3′-tetrahydrofuranylglycine[28] as a novel P_2 substitute for the asparagine side chain in saquinavir (**1**). This inhibitor (compound **9**) reproducibly showed a 4-fold higher potency ($IC_{50} = 0.054 \pm 0.027$ nM) than saquinavir ($IC_{50} = 0.23 \pm 0.1$ nM). Inhibitor **9** ($CIC_{95} = 8$ nM) also showed consistent 3-fold higher CIC_{95} potency over saquinavir ($CIC_{95} = 22$ nM). Further removal of the P_3 quinaldic amide ligand in **9** and incorporation of a stereochemically defined 3-tetrahydrofuran urethane functionality as a P_2 ligand provided inhibitor **10** ($IC_{50} = 160$ nM and $CIC_{50} = 800$ nM). The importance of the cyclic ether was further demonstrated in hydroxyethylene isostere-derived HIV protease inhibitors containing a 3-(*S*)-tetrahydrofuran urethane as the high-affinity P_2 ligand (Figure 2).[29] Inhibitor **11** has shown a 5000-fold enhancement in potency relative to inhibitor **10**.



9: IC_{50} = 0.054 nM
CIC_{95} = 8 nM



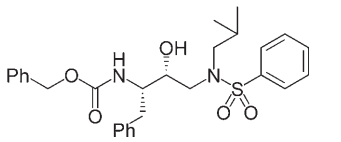
10: IC_{50} = 160 nM
CIC_{95} = 800 nM



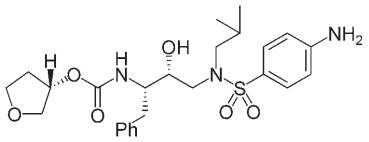
11: IC_{50} = <0.03 nM
CIC_{95} = 3 nM

Figure 2. Hydroxyethylene- and hydroxyethylamine-derived inhibitors.

Researchers at Vertex laboratories developed a significantly lower-molecular-weight protease inhibitor that incorporates 3-(*S*)-tetrahydrofuran as the P_2 ligand and an (*R*)-(hydroxyethyl)-sulfonamide as the isostere **12**.[30] This afforded the highly potent inhibitor VX-476, which was subsequently renamed amprenavir[21] (**7**, Figure 3) and approved by the FDA for the treatment of HIV infection and AIDS. The crystal structure of amprenavir-bound HIV protease revealed the extensive interactions



12: K_i = 3.2 nM
EC_{50} = 113 nM



7: amprenavir
K_i = 0.6 nM
IC_{90} = 40 nM

Figure 3. Urethane-based HIV protease inhibitors.

of the ring oxygen atom of the 3-(*S*)-tetrahydrofuryloxy group. This O atom is apparently involved in a weak interaction with the Asp 29 and Asp 30 backbone amides (at 3.4 and 3.5 Å, respectively).

Development of Bis-THF as a privileged P_2 Ligand

Our structure-based design effort led to the development of a number of cyclic-ether-derived nonpeptide P_2 ligands for the HIV protease substrate binding site. Particularly notable is the potency-enhancing effect of the (3*S*)-tetrahydrofuranyl urethane in inhibitors that contain a hydroxyethylene or a hydroxyethylsulfonamide isostere. As mentioned above, a protein–ligand X-ray crystal structure indicated hydrogen bonding between the tetrahydrofuran (THF) group and the main-chain aspartic acids (Asp 29 and Asp 30), as well as van der Waals interactions in the S_2 site. Our design effort then concentrated on further improving the potency of inhibitor **10** (Figure 2) which contains (3*S*)-tetrahydrofuranyl urethane in the saquinavir-derived hydroxyethylamine isostere. Our objective was to design a ligand that would maximize the hydrophobic and hydrogen bonding interactions with the residues in the S_2 site. To this end, we further explored the use of a polyether template to mimic the peptide region that binds to the viral enzyme. After careful analysis of the X-ray crystal structure of the saquinavir-bound protease, we speculated that exchange of the (3*S*)-tetrahydrofuran moiety for a fused bicyclic tetrahydrofuran (bis-THF) derivative could effectively hydrogen bond to the NH groups of Asp 29 and Asp 30. The conformationally constrained bis-THF should also offset the loss of P_3 hydrophobic binding of the quinoline ring in saquinavir. However, with the ultimate goal of producing high-affinity P_2 ligand, we actually designed and synthesized a new class of cyclic fused bis-THF[31] urethane-based HIV protease inhibitors. The inhibitor **13**, which incorporates (3*R*,3a*S*,6a*R*)-bis-THF as the P_2 ligand, exhibited a significant improvement in enzyme inhibitory and antiviral potency. Inhibitor **13** has shown enzyme inhibitory activity (IC_{50}) of 1.8 nM and a CIC_{95} value of 46 nM (Figure 4). Furthermore, inhibitor **13** has shown improved aqueous solubility, decreased

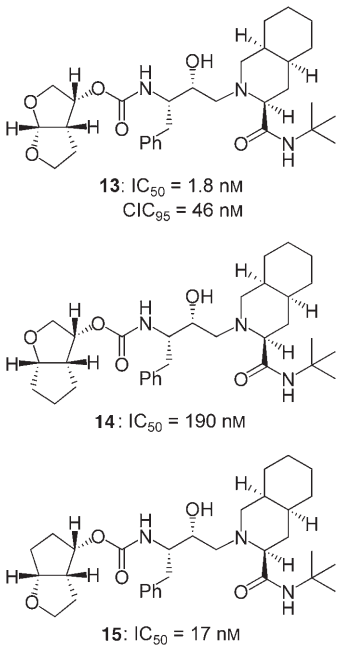


Figure 4. Bis-THF–urethane-based HIV protease inhibitors.

log*P* values, and is lower in molecular weight than saquinavir. Detailed SAR studies indicate that stereochemistry, placement of the oxygen atoms, ring size, and substituents are all essential to optimum binding.

Compared with THF-based inhibitor **10**, bis-THF inhibitor **13** showed nearly 90-fold enhancement in its inhibitory potency and greater than 15-fold enhancement in its antiviral potency. The X-ray crystal structure of **13**-bound protease provided important molecular insight into the ligand binding site interactions. In particular, the bis-THF ring oxygen atoms effectively participate in the same binding site as the P_2 asparagine carboxamide and the P_3 quinaldic amide carbonyl groups of inhibitor **1**. As expected, both oxygen atoms in the bis-THF ligand are involved in hydrogen bonding interactions with the Asp 29 and Asp 30 NH groups present in the S_2 subsite of the protease.

Our initial synthesis of the optically active bis-THF ligand from (*R*)-malic acid was far from satisfactory for carrying out detailed SAR studies.[31a] Our subsequent three-step synthesis of racemic bis-THF followed by lipase-catalyzed efficient optical resolution broaden the scope and utility of this novel polyether-like nonpeptide ligand.[32] Incidentally, the bis-THF ligand is also a subunit of ginkgolides A–C, an important class of natural products with significant biological activities.[33,34] Other economical syntheses of bis-THF ligands have been reported in recent years.[35]

Development of UIC-94003 (TMC-126) and UIC-94017 (TMC-114)

Following discovery of the bis-THF ligand while replacing two amide bonds and the 10π-quinaldic acid amide of saquinavir, we investigated the potential of this ligand in conjunction with a hydroxyethyl(sulfonamide) isostere. As shown in Figure 5, incorporation of (3*R*,3a*S*,6a*R*)-bis-THF as a P_2 ligand and *p*-methoxybenzenesulfonamide as the P_2' ligand provided

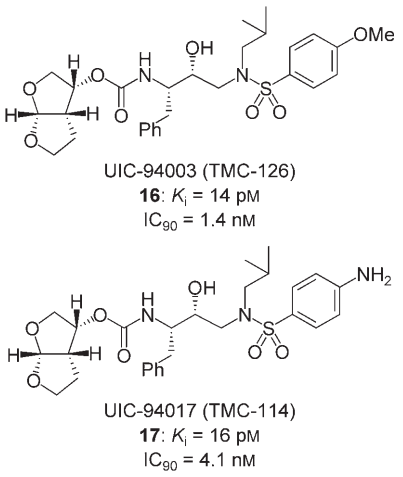


Figure 5. Bis-THF-derived inhibitors TMC-126 and TMC-114.

inhibitor **16** (UIC-94003, $K_i = 14$ pM, $IC_{90} = 1.4$ nM). Similarly, incorporation of a P_2' *p*-aminobenzenesulfonamide provided inhibitor **17** (UIC-94017, $K_i = 16$ pM, $IC_{90} = 4.1$ nM). Both inhibitors exhibited remarkable enzyme inhibitory and antiviral properties.[36]

Inhibitors **16** and **17** were subsequently named TMC-126 and TMC-114, respectively. The in vitro drug-sensitivity studies using HIV-1 laboratory isolates indicated that **16** is one of the most potent inhibitors of wild-type HIV protease. In addition, it was shown to be potent against a wide spectrum of recombinant HIV protease-containing HIV-1 variants that were highly cross-resistant to one or more of the protease inhibitors used in first-line therapy. As can be observed in Table 1, the initial in vitro drug-sensitivity study of **16** with the HIV-1 laboratory isolates HIV-1_{LAI} and HIV-$1_{Ba\text{-}L}$ in PHA-PBMC, or HIV-2_{EHO} in MT-2 cells showed that **16** was >10-fold more potent than five currently available protease inhibitors (RTV, IDV, SQV, NFV, and APV) against HIV-1_{LAI} and HIV-$1_{Ba\text{-}L}$ ($IC_{50} = 0.3$ nM).[37]

Inhibitor **16** also exhibited remarkably potent and unprecedented broad-spectrum activity against a wide range of primary, multidrug-resistant HIV-1 strains isolated from patients with AIDS who had failed 9 to 11 anti-HIV-1 drugs. The results are shown in Table 2. These HIV-1 strains contained 9–14 amino acid substitutions in the protease-encoding region and are known to exert resistance against currently approved protease inhibitors (RTV, IDV, SQV, NFV, and APV). As can be observed in Table 2, all strains had a higher level of resistance (6- to >77-fold) to RTV, IDV, NFV, and APV than the wild-type clinical strain HIV-$1_{ERS104pre}$. Very impressively, TMC-126 suppressed all eight isolates with IC_{50} values ranging from 0.5 to 5 nM.

The inhibitory activity of **16** against wild-type HIV-1 and various mutant proteases is given in Table 3. Vitality was determined from the measured K_i values, and the data indicate that inhibitor **16** possesses highly potent and unprecedented broad-spectrum antiretroviral activity.[38]

Table 1. Sensitivity of HIV-1_{LAI}, HIV-$1_{Ba\text{-}L}$, and HIV-2_{EHO} to various PIs.

Virus	Cell	IC_{50} [nM][a] Ritonavir (RTV)	Indinavir (IDV)	Saquinavir (SQV)	Nelfinavir (NFV)	Amprenavir (APV)	UIC-94003 (TMC-126)
HIV-1_{LAI}	PBMC	40 ± 0.8	15 ± 0.4	11 ± 0.5	9 ± 0.03	17 ± 0.3	0.3 ± 0.09
HIV-$1_{Ba\text{-}L}$	PBMC	38 ± 2	17 ± 0.1	14 ± 1	3 ± 0.2	23 ± 0.9	0.3 ± 0.04
HIV-1_{LAI}	MT-2	41 ± 0.5	19 ± 0.9	23 ± 0.2	5 ± 0.2	41 ± 1	0.3 ± 0.1
HIV-2_{EHO}	MT-2	350 ± 2.5	10 ± 0.4	4 ± 0.05	20 ± 1	530 ± 3	0.5 ± 0.07

[a] Data represent mean values ± SD derived from the results of three independent experiments conducted in duplicate or triplicate. For PBMC (peripheral blood mononuclear cells), IC_{50} values were determined by using PHA-PBMC exposed to each HIV-1 preparation ($50 \times TCID_{50}$ per 10^5 PBMC) in the presence of each anti-HIV-1 agent and by using the inhibition of p24 Gag protein production as an endpoint on day 7 of culture ($TCID_{50}$ = 50% tissue culture infective dose). MT-2 cells (2×10^3) were exposed to 100 $TCID_{50}$ of HIV-1_{LAI} or HIV-2_{EHO} and cultured in the presence of various concentrations of PIs, and the IC_{50} values were determined using the MTT assay on day 7 of culture. (MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.)

Upon selection of HIV-1 in the presence of TMC-126, mutants carrying a novel active site mutation A28S appeared along with L10F, M46I, I50V, A71V and N88V. The drug-sensitivity results of TMC-126-selected HIV-1 strains to PIs are described in Table 4. These results indicate that with IC_{50} values as low as 0.02 μM, TMC-126 suppressed the replication of HIV-1 variants selected with 62 and 30 passages in the presence of increasing concentrations of TMC-126 and amprenavir, respectively. Our detailed studies and data have provided firm evidence that TMC-126 has significant advantages over other protease inhibitors.

Inhibitor **17**, which is structurally related to **16**, has also shown similar antiviral activity and resistance profiles. However, **17**, with a basic P_2' amine functionality, has shown favorable pharmacokinetic properties in animals. It was subsequently selected for clinical development and underwent multicenter clinical trials.[39]

Recently, we demonstrated that **17** exerts more potent activity against a laboratory HIV-1 strain, HIV-1_{LAI}, relative to the activities of the currently available FDA-approved protease inhibitors (Table 5). The six available protease inhibitors (SQV, APV, IDV, NFV, RTV, and LPV) suppressed the infectivity and replication of HIV-1_{LAI}, with IC_{50} values ranging from 0.017 to 0.047 μM in MT-2 cells, whereas TMC-114 had the most potent activity in terms of suppressing the infectivity and replication of the virus (IC_{50} = 0.003 μM).[40]

Inhibitor **17** was further tested against R5 laboratory HIV-1 strain, HIV-$1_{Ba\text{-}L}$, and two HIV-2 strains, HIV-2_{ROD} and HIV-2_{EHO} in vitro. It was also found that **17** had greater activities (6- to 13-fold) against HIV-$1_{Ba\text{-}L}$ than the other tested protease inhibitors. In addition, **17** had more potent activity than the other four protease inhibitors against two HIV-2 strains, suppressing their infectivity and replication (Table 6).

Table 3. Enzyme inhibitory potency of **16** against wild-type and mutant proteases.

Enzyme	K_i [pM]	$K_{i(WT)}/K_{i(mutant)}$	Vitality
WT	14	1	1
D30N	< 5	0.33	0.3
V32I	8	0.57	0.5
I84V	40	2.85	1
V32I/I84V	70	5	0.7
M46F/V82A	< 5	0.33	0.1
G48V/L90M	< 5	0.33	0.1
V82F/I84V	7	0.5	0.1
V82T/I84V	22	1.57	0.1
V32I/K45I/F53L/A71V/I84V/L89M	31	2.2	0.1
V32I/L33F/K45I/F53L/A71V/I84V	46	3.3	0.1
K20R/M36I/I54V/A71V/V82T	31	2.2	0.1

Table 2. PI sensitivity of HIV-1 isolated from heavily drug-experienced individuals.

Virus	Amino Acid Substitution[a]	IC_{50} [μM][c] RTV	IDV	SQV	NFV	APV	TMC-126
WT[b]	L63P	0.044 (1)	0.013 (1)	0.010 (1)	0.023 (1)	0.025 (1)	0.0007 (1)
1	L10I, K14R, L33I, M36I, M46I, F53L, K55R, I62V, L63P, A71V, G73S, V82A, L90M, I93L	> 1 (> 23)	> 1 (> 77)	0.27 (27)	> 1 (> 43)	0.27 (11)	0.004 (6)
2	L10I, I15V, K20R, M36I, M46L, I54V, K55R, I62V, L63P, K70Q, V82A, L89M	> 1 (> 23)	0.49 (38)	0.037 (4)	0.33 (14)	0.28 (11)	0.0013 (2)
3	L10I, I15V, E35D, N37E, K45R, I54V, L63P, A71V, V82T, L90M, I93L, C95F	> 1 (> 23)	0.49 (38)	0.036 (4)	> 1 (> 43)	0.26 (10)	0.001 (1)
4	L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, L90M	> 1 (> 23)	0.21 (16)	0.033 (3)	0.09 (4)	0.31 (12)	0.0016 (2)
5	L10I, K43T, M46L, I54L, L63P, A71T, V82A, L90M, Q92K	> 1 (> 23)	> 1 (> 77)	0.31 (31)	0.41 (18)	0.67 (27)	0.0024 (3)
6	L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M, I93L	> 1 (> 23)	0.30 (23)	0.19 (19)	> 1 (> 43)	0.16 (6)	0.0005 (1)
7	L10I, L24I, L33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, V82A	> 1 (> 23)	> 1 (> 77)	0.12 (12)	> 1 (> 43)	0.49 (20)	0.0055 (8)
8	L10R, N37D, M46I, I62V, L63P, A71V, G73S, V77I, V82T, L90M, I93L	> 1 (> 23)	0.55 (42)	0.042 (4)	> 1 (> 43)	0.15 (6)	0.001 (1)

[a] In PR. The amino acid sequence of each viral isolate was deduced from the nucleotide sequence and compared with the consensus B sequence cited from the Los Alamos data base. [b] A clinical isolate, HIV-$1_{ERS104pre}$, served as a source of wild-type (WT) HIV-1. [c] IC_{50} values were determined by using PHA-PBMC exposed to HIV-1 strains ($50 \times TCID_{50}$ per 10^5 PBMC) in the presence of each anti-HIV-1 agent and by using the inhibition of p24 Gag protein production as an endpoint. All values were determined in triplicate, and those shown are representative of two or three separate experiments. Numbers in parentheses represent the fold change of IC_{50} values against each isolate compared with the IC_{50} against HIV-1 wild-type.

Table 4. Amino acid substitution in PR and sensitivity of drug-resistant HIV-1 strains to PIs.[a]

Virus	Amino Acid Substitution	IC_{50} [μM]					
		RTV	IDV	SQV	NFV	APV	TMC-126
$HIV\text{-}1_{NL4\text{-}3}$	-	0.038 (1)	0.011 (1)	0.019 (1)	0.023 (1)	0.042 (1)	0.0003 (1)
$HIV\text{-}1_{UIC\text{-}P62}$	L10F, A28S, M46I, I50V, A71V, N88D	0.055 (1)	0.08 (7)	0.01 (1)	0.11 (5)	0.83 (20)	0.021 (70)
$HIV\text{-}1_{APVP30}$	L10F, V32I, K20R, M36I, M46I, I54M, A71V, I84V	>1 (>26)	0.32 (30)	0.035 (2)	>1 (43)	>1 (>25)	0.029 (100)

[a] MT-2 cells (2×10^3) were exposed to $HIV\text{-}1_{NL4\text{-}3}$, $HIV\text{-}1_{UIC\text{-}P62}$, (HIV-1 following 62 passages in the presence of increasing concentrations of TMC-126), or $HIV\text{-}1_{APV\text{-}P30}$ (HIV-1 following 30 passages in the presence of increasing concentrations of APV, all $100\times TCID_{50}$) and cultured in the presence of various drug concentrations. The IC_{50} values were determined on day 7 of culture in the MTT assay. All values were determined in duplicate, and those shown are representative of two or three independent experiments. The numbers in the parentheses represent fold changes relative to $HIV\text{-}1_{NL4\text{-}3}$ (wild-type).

Table 5. Cytotoxicity and activity of UIC-94017 (TMC-114) against $HIV\text{-}1_{LAI}$.[a]

Drug	IC_{50} [μM]	CC_{50} [μM]	Selectivity Index
TMC-114	0.003 ± 0.0001	74.4 ± 1.2	24 800
SQV	0.017 ± 0.003	11.3 ± 2.8	660
APV	0.036 ± 0.011	>100	>2800
IDV	0.047 ± 0.008	70.3 ± 4.6	1500
NFV	0.027 ± 0.004	ND	ND
RTV	0.045 ± 0.012	ND	ND
LPV	0.034 ± 0.006	ND	ND

[a] MT-2 cells (2×10^3) were exposed to $100\times TCID_{50}$ of $HIV\text{-}1_{LAI}$ and cultured in the presence of various concentrations of PIs. The IC_{50} values were determined with the MTT assay on day 7 of culture. All assays were conducted in duplicate, and the data shown represent the mean ± SD from the results of three independent experiments (ND: not determined). [b] Concentration that causes 50% cytotoxicity.

Further tests on the activity of **17** and the five clinically available PIs revealed that **17** effectively blocked the infectivity and replication of each of the $HIV\text{-}1_{NL4\text{-}3}$ variants exposed to and selected for resistance to SQV, IDV, NFV, or RTV at concentrations up to 5 μM (Table 7).[40] Also, **17** exerted potent activity against highly multi-PI-resistant clinical HIV-1 variants isolated from seven patients with AIDS who had no response to existing antiviral regimens after having received a variety of antiviral agents (Table 8).

One of our guiding principles to combat drug resistance is to design ligands and incorporate structural features in the inhibitors to maximize the interactions in the active site of HIV protease. In particular, we strive toward making extensive hydrogen bonding interactions with the protein backbone. As there is only a small distortion between the backbone conformations of protein–ligand complexes of wild-type HIV protease

Table 6. Activities of selected anti-HIV agents against $HIV\text{-}1_{Ba\text{-}L}$, $HIV\text{-}2_{ROD}$, and $HIV\text{-}2_{EHO}$.

Virus	Cell	IC_{50} [μM][a]						
		AZT	SQV	APV	IDV	NFV	RTV	TMC-114
$HIV\text{-}1_{Ba\text{-}L}$[b]	PBMC	0.009 ± 0.001	0.018 ± 0.010	0.026 ± 0.005	0.025 ± 0.012	0.017 ± 0.004	0.039 ± 0.020	0.003 ± 0.0003
$HIV\text{-}2_{ROD}$[c]	MT-2	0.018 ± 0.002	0.003 ± 0.0002	0.23 ± 0.01	0.014 ± 0.006	0.019 ± 0.003	0.13 ± 0.06	0.003 ± 0.0001
$HIV\text{-}2_{EHO}$[c]	MT-2	0.011 ± 0.002	0.006 ± 0.002	0.17 ± 0.05	0.011 ± 0.002	0.029 ± 0.018	0.24 ± 0.006	0.006 ± 0.003

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

Table 7. Activity of TMC-114 against laboratory PI-resistant HIV-1.

Virus	Amino Acid Substitution[a]	IC_{50} [μM][b]					
		SQV	APV	IDV	NFV	RTV	TMC-114
$HIV\text{-}1_{NL4\text{-}3}$	WT	0.009 (1)	0.027 (1)	0.011 (1)	0.020 (1)	0.018 (1)	0.003 ± 0.0005 (1)
$HIV\text{-}1_{SQV5\mu M}$	L10I, G48V, I54V, L90M	>1 (>111)	0.17	>1 (>91)	0.30 (15)	>1 (>56)	0.005 ± 0.0009 (2)
$HIV\text{-}1_{APV5\mu M}$	L10F, V32I, M46I, I54M, A71V, I84V	0.020 (2)	>1 (>37)	0.31 (28)	0.21 (11)	>1 (>56)	0.22 ± 0.05 (73)
$HIV\text{-}1_{IDV5\mu M}$	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)
$HIV\text{-}1_{NFV5\mu M}$	L10F, D30N, K45I, A71V, T74S	0.031 (3)	0.093 (3)	0.28 (25)	>1 (>50)	0.09 (5)	0.003 ± 0.0002(1)
$HIV\text{-}1_{RTV5\mu M}$	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 ($100\times TCID_{50}$), and the inhibition of p24 Gag protein production by the drug was used as an endpoint. Numbers in parentheses represent the fold change in IC_{50} for each isolate relative to that of $HIV\text{-}1_{NL4\text{-}3}$. The data represent the mean ± SD from the results of three independent experiments conducted in triplicate.

Table 8. Activity of TMC-114 against HIV-1 clinical isolates in PHA-PBMC.[a]

Virus	IC_{50} [μM]						
	AZT	SQV	APV	IDV	NFV	RTV	TMC-114
HIV-$1_{ERS104pre}$ (WT X4)	0.004	0.010	0.023	0.018	0.019	0.027	0.003
HIV-1_{MOKW} (WT R5)	0.016	0.004	0.011	0.018	0.033	0.032	0.003
HIV-1_{TM} (MDR X4)	0.73 (183)	0.23 (23)	0.39	>1 (>56)	0.54 (28)	>1 (>37)	0.004 (1)
HIV-1_{MM} (MDR R5)	0.37 (93)	0.30 (30)	0.34	>1 (>56)	>1 (>53)	>1 (>37)	0.02 (7)
HIV-1_{JSL} (MDR R5)	0.08 (20)	0.35 (35)	0.75 (33)	>1 (>56)	>1 (>53)	>1 (>37)	0.029 (10)
HIV-1_{A} (MDR X4)	ND	0.14 (14)	0.16 (7)	>1 (>56)	0.36 (19)	>1 (>37)	0.004 (1)
HIV-1_{B} (MDR X4)	ND	0.31 (31)	0.34 (15)	>1 (>56)	>1 (>53)	>1 (>37)	0.013 (4)
HIV-1_{C} (MDR X4)	ND	0.037 (4)	0.28 (12)	>1 (>56)	0.44 (23)	>1 (>37)	0.003 (1)
HIV-1_{G} (MDR X4)	ND	0.029 (3)	0.25 (11)	0.39 (22)	0.32 (17)	0.44 (16)	0.004 (1)

[a] The amino acid substitutions identified in the PR-encoding region of HIV-$1_{ERS104pre}$, HIV-1_{TM}, HIV-1_{MM}, HIV-1_{JSL}, HIV-1_{A}, HIV-1_{B}, HIV-1_{C}, and HIV-1_{G} relative to the consensus type B sequence cited from the Los Alamos database include: L63P (HIV-$1_{ERS104pre}$); L10I, K14R, R41K, M46L, I54V, L63P, A71V, V82A, L90M, and I93L (HIV-1_{TM}); L10I, K43T, M46L, I54V, L63P, A71V, V82A, L90M, and Q92K (HIV-1_{MM}); L10I, L24I, L33F, E35D, M36I, N37S, M46L, I54V, R57K, I62V, L63P, A71V, G73S, and V82A (HIV-1_{JSL}); L10I, I15V, E35D, N37E, K45R, I54V, L63P, A71V, V82T, L90M, I93L, and C95F (HIV-1_{A}); L10I, K14R, L33I, M36I, M46I, F53I, K55R, I62V, L63P, A71V, G73S, V82A, L90M, and I93 (HIV-1_{B}); L10I, I15V, K20R, L24I, M36I, M46L, I54V, I62V, L63P, K70O, V82A, and L89M (HIV-1_{C}); and L10I, V11I, T12E, I15V, L19I, R41K, M46L, L63P, A71T, V82A, and L90M (HIV-1_{G}). HIV-1_{MOKW} was confirmed to lack any known drug-resistance-associated amino acid substitutions. IC_{50} values were determined by using PHA-PBMC as target cells and the inhibition of p24 Gag protein production as an endpoint. All values were determined in triplicate, and those shown are derived from the results of three independent experiments. Numbers in parentheses represent the fold change in IC_{50} values for each isolate relative to those of HIV-$1_{ERS104pre}$. MDR = multidrug resistant; ND = not determined.

and mutant HIV proteases,[41] it is conceivable that such backbone hydrogen bonding interactions can be maintained with the mutant proteases. Our design of inhibitor **17** is based on this hypothesis. To investigate the mechanism by which **17** exerts its potent activity against a wide spectrum of multi-PI-resistant HIV-1 strains, an X-ray crystal structure of HIV-1 protease complexed with **17** at 1.30 Å resolution was examined.[42] It was found that the two oxygen atoms of the bis-THF groups of **17** formed strong hydrogen bonds with the main chains of Asp 29 and Asp 30 in the S_2 subsite (Figure 6). It was also found that **17** formed new polar interactions with the amide of the main chain and the carboxylate oxygen atom of Asp 30. These interactions are proposed to be crucial and could be the reason for potent activity against multi-PI-resistant variants.[43] Its highly potent antiviral activity against wild-type HIV-1 isolates and a large panel of PI-resistant viruses, as well as its pharmacokinetic properties made inhibitor **17** the choice as a candidate for development and further clinical studies. Inhibitor **17** has subsequently been renamed darunavir.

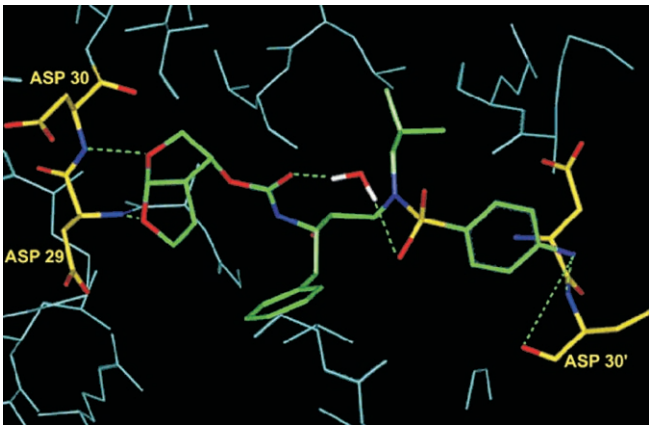


Figure 6. Hydrogen bond interactions of HIV protease with **17** (TMC-114, darunavir).

Preclinical Results of TMC-114 (Darunavir)

TMC-114 exhibited the following characteristics in our assay: antiviral $IC_{50} = 4.7$ nM, $IC_{90} = 10.3$ nM, $CC_{50} > 100$ μM in a cell culture assay. TMC-114 was tested against a panel of 20 HIV variants resistant to current protease inhibitors, but there was no greater than a 5-fold increase in IC_{50} values. The observed IC_{50} values were < 100 nM against 100% ($IC_{50} < 10$ nM against 94%) of 261 randomly selected, recent recombinant clinical isolates, among which 32% show a ≥ 10-fold increase in IC_{50} for at least one of the current PIs. Good relative stability upon incubation with human liver microsomes was demonstrated. This inhibitor maintains high blood levels in dogs at an oral dose of 20 mg kg^{-1} body mass (C_{max} plasma concentrations of 13.7 μM). It has shown excellent potency ($IC_{50} < 10$ nM) against clinical HIV-1 isolates that exhibit resistance to currently approved protease inhibitors.[44]

A randomized, double-blind, placebo-controlled, dose-escalating trial was performed to examine the safety, tolerability, and pharmacokinetics of single oral doses of inhibitor **17** (TMC-114). Two panels of nine healthy volunteers (six active, three placebo) received alternating doses of 100, 200, 400, 800, 1200, or 1600 mg. Because the maximum tolerated dose was not reached, additional doses were added to administer 2400, 3200, and 4000 mg. Initially, plasma concentrations increased greater than proportional with the dosing. No further increases were observed between 2400 and 3200 mg. The mean C_{max} was 14.4–15.3 μg mL^{-1} (26.2–27.8 μM) at these dose levels. The elimination half-life was approximately 10 h, irrespective of dose. For doses of 800 mg and greater, plasma levels at 8–12 h post-dose exceeded protein-adjusted IC_{50} values for isolates resistant to currently approved PIs. All doses were considered safe. Diarrhea, related to polyethylene glycol (PEG) in the formulation, occurred at high dose levels and limited further escalation. Short-term localized paresthesia (oral, 3; fingers, 1) was observed in four out of six subjects at the 3200 mg dose. These studies demonstrated that single doses of **17** were safe and well-tolerated at all doses tested. The maximum tolerated dose was not achieved. Further dose increases were hindered by solvent-related diarrhea. Single-dose plasma levels provided superior inhibitory quotients for PI-resistant HIV-1 isolates over

currently approved protease inhibitors. Tibotec (Belgium) has carried out clinical development of darunavir (TMC-114).[39] Recently, the FDA has approved darunavir for treatment of drug-resistant HIV.[45]

Recently, researchers at Tibotec made the effort to confirm and further examine the antiviral activity (against both wild-type and PI-resistant HIV), cytotoxicity, and mechanism of action of TMC-114.[46] The results of in vitro studies of TMC-114 against different laboratory HIV strains revealed potent anti-HIV activity, with IC_{50} values in the range of 1–5 nM and corresponding IC_{90} values in the range of 2.7–13 nM. In terms of cytotoxicity, TMC-114 exhibited no cytotoxicity at concentrations up to 100 μM, and the selectivity index was found to be $>20\,000$ for wild-type HIV-1. In addition, TMC-114 was equally active against 32 recombinant strains from clinical isolates. The effect of human serum and alpha-1-acid glycoprotein (AAG) on the antiviral activity of TMC-114 and other approved PIs at 0.5–5 μM showed a <7-fold decrease in potency, pointing to a saturable binding of PIs to AAG. The activity studies against PI-resistant HIV-1 variants, in a panel of 17 recombinant clinical isolates carrying multiple protease mutations and demonstrating resistance to an average of five other PIs, were susceptible to TMC-114, defined as a fold change in IC_{50} of <4. TMC-114 was also effective against the majority of 1501 PI-resistant recombinant viruses derived from recent clinical samples with IC_{50} values of <10 nM for 75% of the samples. Isothermal titration calorimetry also showed very high-affinity binding (K_d = 0.0045 nM) of TMC-114 to HIV-1 protease. X-ray crystallographic analysis confirmed that TMC-114 forms strong hydrogen bonds with residues in the main chain of the protease active site (Asp 29 and Asp 30).[42]

Exploration of P_2' Ligand Functionalities

We further incorporated a number of other functionalities at the P_2' sulfonamide to interact specifically with residues in the enzyme active site. The results are reported herein for the first time.[38a] Based on the X-ray crystal structure of HIV-1 protease bound to inhibitor **1**, various functionalities on the sulfonamide ligands can form hydrogen bonds with the backbone of Asp 29′ and Asp 30′. The X-ray crystal structure of HIV-1 protease bound to inhibitor **13** shows that, in some cases, there are favorable interactions with the side chain of Asp 30′ as well.

A series of inhibitors (**18**–**22**) in Figure 7 have also shown exceedingly potent enzyme inhibition properties. Inhibitors **19**, **20**, and **21** were tested against proteases containing the noxious drug resistance associated mutations V82F/I84V and G48V/V82A. These inhibitors also possess broad-spectrum potent activity against mutant proteases. Inhibitor **22**, with a benzodioxanesulfonamide derivative as the P_2' ligand, has also exhibited marked enzyme inhibitory potency (<5 pM) and antiviral potency (IC_{50} = 1.1 nM in MT-2 cells). The antiviral potency of inhibitors **19**–**22** was determined with respect to wild-type clinical isolates HIV-1_{LAI} and HIV-$1_{Ba\text{-}L}$. The latter is a monocytotropic strain of HIV. The IC_{50} values for isolates HIV-1_{LAI} and HIV-$1_{Ba\text{-}L}$ were determined by exposing the PHA-simulated PBMC to

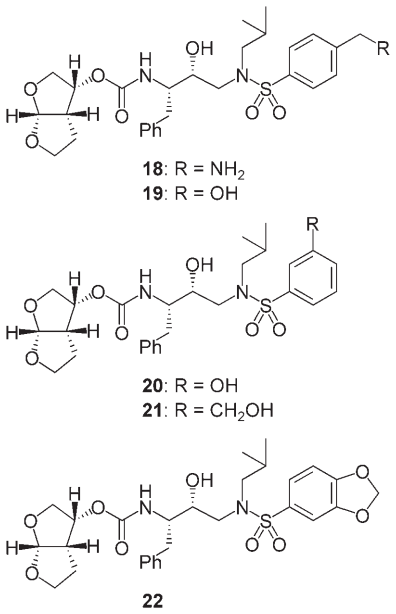


Figure 7. Bis-THF-based P_2' sulfonamide inhibitors.

HIV-1 ($50\times TCID_{50}$ dose per 1×10^6 PBMC) in the presence of various concentrations of inhibitors **19**–**22**; the inhibition of p24 *gag* protein production was used as an endpoint on day 7 of culture ("p24 assay"). All drug sensitivities were performed in triplicate. The IC_{50} values for isolate HIV-1_{LAI} were also determined by exposing MT-2 cells (2×10^3) to $100\times TCID_{50}$ of HIV-1_{LAI} cultured in the presence of various concentrations of PIs.[47] The IC_{50} values were determined using the MTT assay on day 7 of culture. All sensitivities were determined in duplicate. The results are shown in Table 9. Thus, it appears that inhibitor **22** may exhibit a similar level of potency as inhibitor **16** (TMC-126) in various multidrug-resistant HIV strains.

Table 9. Antiviral potency of PIs **18**–**22**.

			IC_{50} [nM]				
Virus	Cell Type	Assay	**18**	**19**	**20**	**21**	**22**
HIV-1_{LAI}	MT-2	MTT	28	5.3	17	28	0.22
HIV-1_{LAI}	PBMC	p24	20	2.7	34	8	0.22
HIV-$1_{Ba\text{-}L}$	PBMC	p24	13	3	38	9.3	0.33

Bis-THF-Derived New Generation of HIV-1 Protease Inhibitors

Because of its extraordinary potency-enhancing effect and its ability to maintain potency against multi-PI-resistant isolates, the bis-THF ligand was incorporated into other isosteres. Abbott research group has recently disclosed the modification of ritonavir by incorporating bis-THF as the P_2 ligand.[48] The SAR studies of the conformationally constrained bis-THF P_2 ligand in combination with a dimethylphenoxyl acetate as a P_2' ligand yielded a series of potent HIV protease inhibitors, of which compounds **23** and **24** (Figure 8) have shown EC_{50} values of 31 and 76 nM, respectively, in the presence of human serum (50%).[48a]

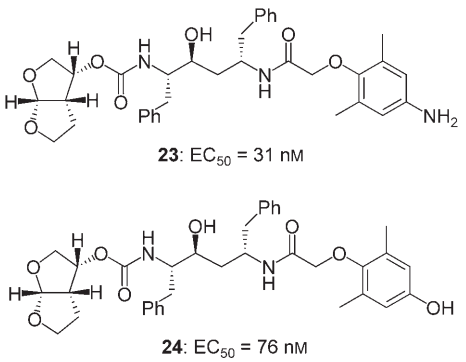


Figure 8. Abbott inhibitors with bis-THF in ritonavir isosteres.

GlaxoSmithKline researchers attempted further optimization of a hydroxyethylsulfonamide series of inhibitors by altering the substitutions on the P_1 and P_1' chains.[49] The structural modifications at the P_1' position resulted in highly potent molecules both in enzyme inhibition and antiviral assays. As shown in Figure 9, inhibitors **25** and **26** have shown enzyme inhibitory potency in the single-digit femtomolar (fM) range (**25** $K_i = 10$ fM and **26** $K_i = 100$ fM, Figure 9). These inhibitors also exhibited impressive antiviral potency.[49a]

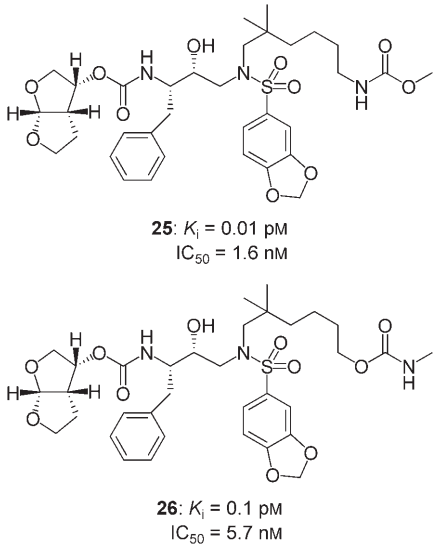


Figure 9. GlaxoSmithKline inhibitors with bis-THF and P_1' modification.

GlaxoSmithKline researchers further explored the structural modification of the P_1 ligand. They investigated tyrosine-derived inhibitors to achieve additional ligand–enzyme interactions. A number of remarkably potent inhibitors emerged from this investigation. As shown in Figure 10, inhibitors **27** and **28** have shown femtomolar enzyme inhibitory activity and very impressive antiviral activity.[50] However, SAR studies suggested that their activities are probably more a function of physicochemical parameters than any specific P_1 side-chain binding interactions. The heteroarylmethyl class of inhibitors **27** and **28** afforded the best activities overall, with single-digit nanomolar IC_{50} values against wild-type HIV virus (HXB2) and two multi-PI-

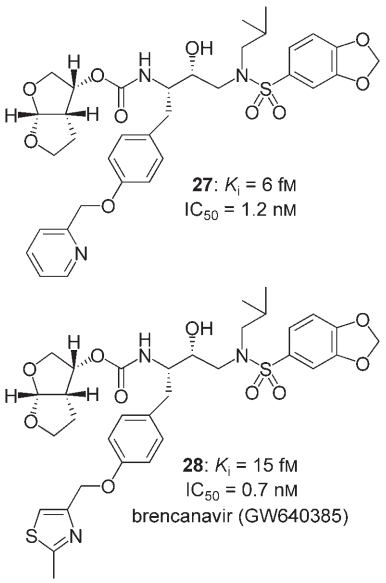


Figure 10. GlaxoSmithKline's bis-THF-based P_1 aryl derivatives.

resistant viruses (EP13 and D545701) in an MT-4 cell line. Compounds **27** and **28** were also found to have K_i values against wild-type HIV protease of 6 and 15 fM, respectively, which make these inhibitors 2400–6000-fold more potent than amprenavir.

As shown in Table 10, inhibitor **28** (GW640385) exhibited antiviral activity (IC_{50}) values of 0.7, 4.8, and 1.1 nM against HXB2, D545701, and EP13 viral strains, respectively.[50] The bioavailability of thiazole derivative **28** yielded 10% *F* and 20% *F* in rat and dog, respectively. Co-administration of 4 mg kg^{-1} of ritonavir with **28** improved oral bioavailability in rat and dog to 62% *F* and 86% *F*, respectively. These results led to the selection of GW640385 as a new clinical candidate.[50] Subsequently, **28** has been renamed brecanavir. Brecanavir has now advanced to Phase-III clinical development.

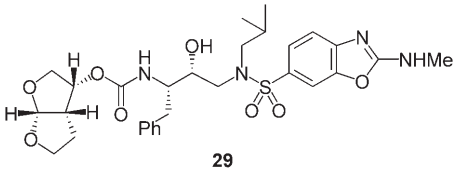
Tibotec researchers have extensively investigated TMC-126 (**16**) and TMC-114 (**17**).[51] Their efforts in modification of the P_2' sulfonamide ligand of TMC-114 led to the discovery of a new series of fused benzoxazole **29** and benzothiazole **30** sulfonamides (Figure 11).[52] Benzothiazole and benzoxazole inhibitors showed improved broad-spectrum antiviral activity in the range of 7.5–8.0 (pEC_{50}) against highly PI-resistant mutants.[52] Selected compounds have shown improved oral bioavailability (in silico and in vitro), solubility at different pH, permeability in Caco-2 assays, and metabolic stability in the presence of rat, dog, and human liver microsomes. Crystal structure determination, molecular modeling, and in vivo studies in rat and dog were performed to rationalize the broad-spectrum profiles of the antiviral activity of benzothiazole and benzoxazole inhibitors.

The crystal structure of HIV protease in complex with inhibitor **30** revealed the critical interactions associated with the P_2' surrogate and S_2' domain of the enzyme. The N atom of the thiazole ring in **30** interacts with the backbone NH group of Asp 30′. The secondary amines present in both inhibitors **29**

Table 10. Resistance profile of **28** against a panel of PI-mutant viruses.

Virus	Mutations in PR	IC_{50} [nM][a]
WT HIV-1		0.7
468 APV-resistant	I15V, E34G, M36I, S37E, I50V, L63P	8.2
D545701 multi-PI-resistant	L10I, L19Q, K20R, E35D, M36I, S37N, M46I, I50V, I54V, I62V, L63P, A71V, V82A, L90M	4.8
14330 NFV-resistant	D30N, E35D, M36I, S37D, I62V, L64P, I64M, N88D	0.45
30813 IDV-resistant	I15V, I54V, R57K, I62V, L63P, H69Y, A71T, I72E, V82A, I85V	0.46
31246 multi-PI-resistant	L10I, I54V, L63P, A71V, I72V, V77I, V82A, I84V, L90M, Q92K	1.7
EP13 multi-PI-resistant	M46I, L63P, A71V, V82F, I84V	1.1
EP14 multi-PI-resistant	L10R, M46I, L63P, V82T, I85V	0.77
Triple multi-PI-resistant	M46I, I47V, I50V	5.4
I50V	I50V	1.6
I54V	I54V	0.22

[a] Determined in MT-4 cells.



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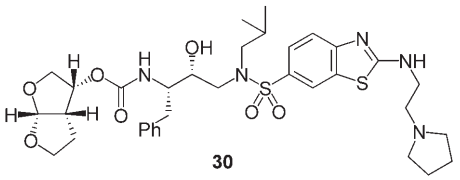

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Figure 11. Tibotec's fused benzoxazole and benzothiazole analogues.

and **30** form a strong hydrogen bond with the side chain of Asp 30′. Moreover, the pyrrolidine ring in inhibitor **30** is in close proximity to form a strong hydrogen bond with the Asp 29′ side chain. These inhibitors are currently undergoing extensive preclinical investigation.

Conclusions

In summary, the structure-based design of bis-tetrahydrofuranyl urethane has emerged as a privileged nonpeptide P_2 ligand for a variety of highly potent HIV-1 protease inhibitors. Incorporation of this ligand provided HIV protease inhibitors with exceedingly potent antiviral activity and superior activity against multi-PI-resistant variants relative to other FDA-approved PIs. Recently, TMC-114 (darunavir) has been approved by the FDA for treatment of drug-resistant HIV. GW640385 (brecanavir), which incorporates bis-THF as the P_2 ligand, is currently in Phase-III clinical development. The bis-THF ligand has been specifically designed to fill in the hydrophobic S_2 pocket effectively and to promote extensive hydrogen bonding with the protein backbone in the enzyme S_2 site. The protein–ligand X-ray crystal structures with TMC-114 and other inhibitors with the bis-THF ligand revealed extensive interactions with the backbone of residues Asp 29 and Asp 30 at the S_2 site. The current design concept targeting the protein backbone may serve as an important guide to combat drug resistance. Further design and synthesis of conceptually novel inhibitors are in progress.

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