ACCOUNTS
of chemical research

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

ARUN K. GHOSH,*,† BRUNO D. CHAPSAL,† IRENE T. WEBER,‡ AND HIROAKI MITSUYA§,⊥

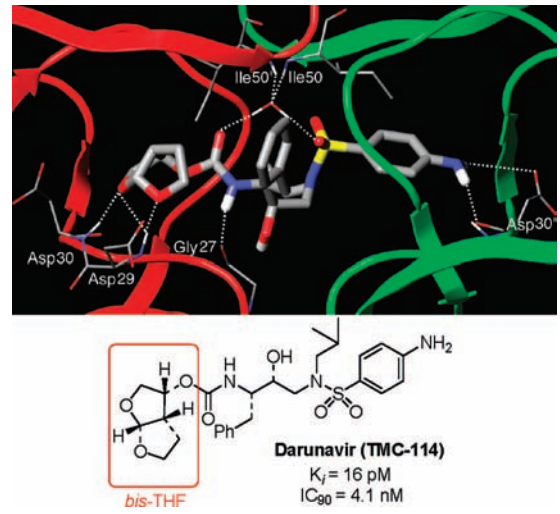
†Departments of Chemistry and Medicinal Chemistry, Purdue University, West Lafayette, Indiana 47907, ‡Department of Biology, Molecular Basis of Disease Program, Georgia State University, Atlanta, Georgia 30303, §Departments of Hematology and Infectious Diseases, Kumamoto University School of Medicine, Kumamoto 860-8556, Japan, and ⊥Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, Bethesda, Maryland 20892

RECEIVED ON MAY 18, 2007

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.

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.

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

Introduction

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

Molecular Insight and Design Strategies To Combat Drug Resistance

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

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

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

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

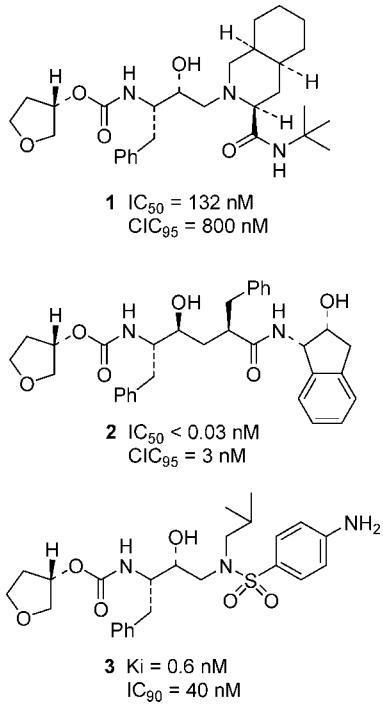


FIGURE 1. Cyclic ether-containing protease inhibitors.

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

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

Synthesis of the Bis-THF Ligand

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

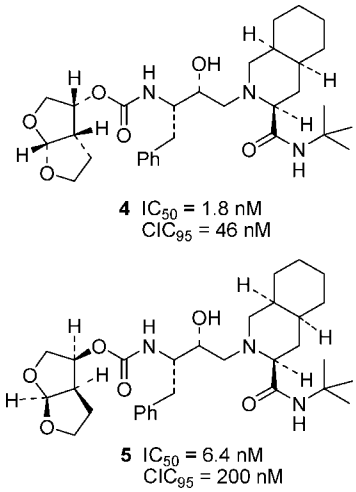


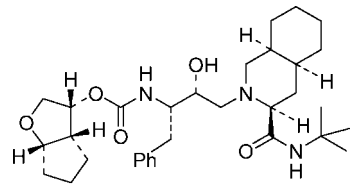
FIGURE 2. Bis-THF-containing protease inhibitors.

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

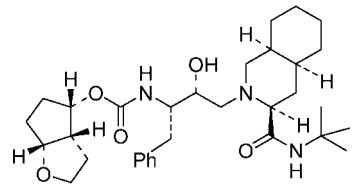
Development of Darunavir

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

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



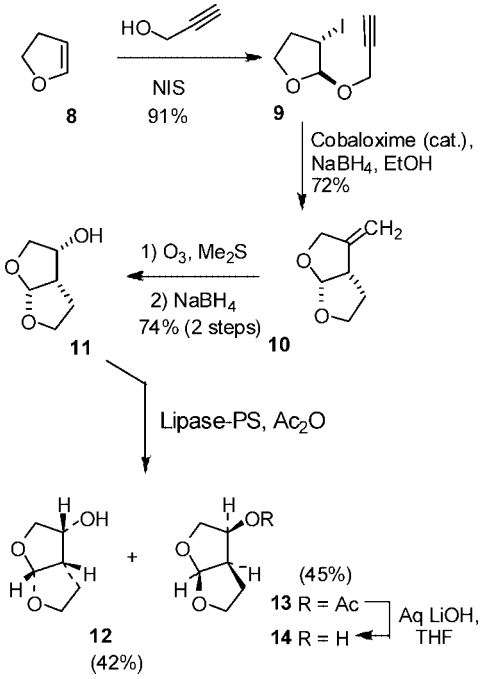
6 IC_{50} = 190 nM



7 IC_{50} = 17 nM

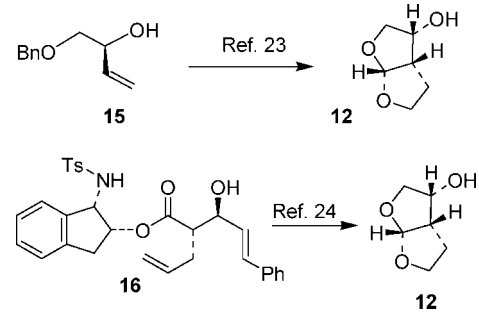
FIGURE 3. Structure of inhibitors **6** and **7**.

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



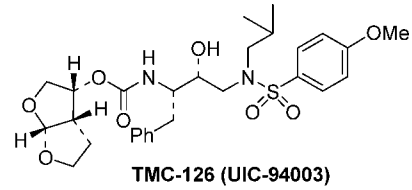
Incorporation of bis-THF into a *p*-aminosulfonamide isostere led to inhibitor **18**. Inhibitor **18** also showed unprecedented antiviral activity and outperformed most of the other currently available PIs against HIV-$1_{Ba\text{-}L}$ by a 6−13-fold difference in IC_{50} values (Table 1).[28] Furthermore,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

SCHEME 2. Stereoselective Syntheses of the Bis-THF Ligand



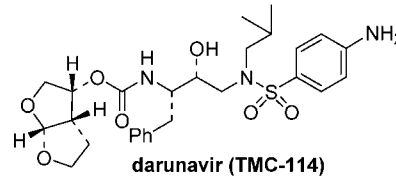
X-ray Crystal Structure of Darunavir and Evidence of Backbone Binding

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



TMC-126 (UIC-94003)

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



darunavir (TMC-114)

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

FIGURE 4. Bis-THF-Derived Protease inhibitors.

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

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

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

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

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

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

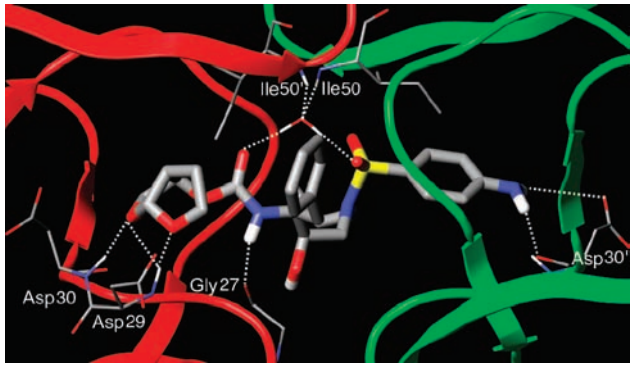


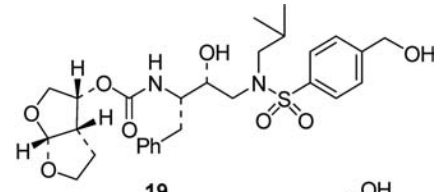
FIGURE 5. Interactions in X-ray crystal structure of **18**-bound HIV protease.

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

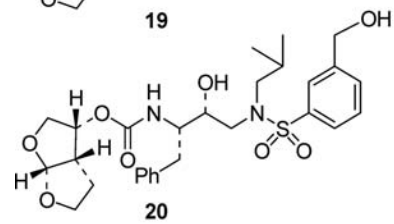
Clinical Development of Darunavir

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

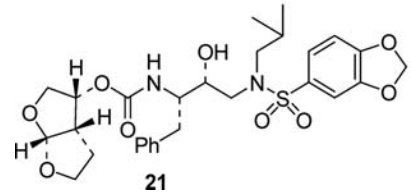
TABLE 3. Sensitivity of HIV-1_{LAI} and HIV-$1_{Ba\text{-}L}$ against New PIs



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-$1_{Ba\text{-}L}$[b]	PBMC	p24	3	9.3	0.33

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

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

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

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

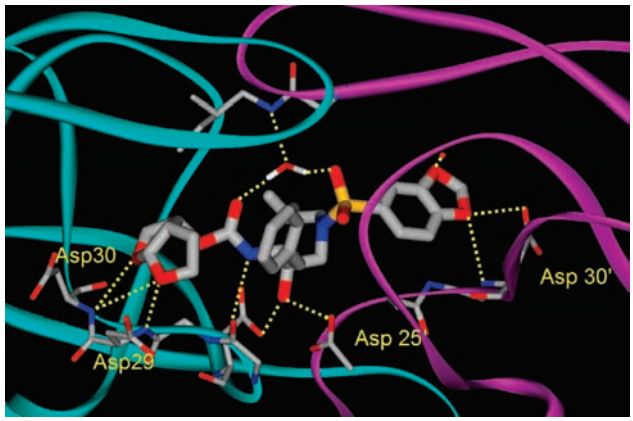


FIGURE 6. Crystal structure of inhibitor **21**-bound HIV-1 protease.

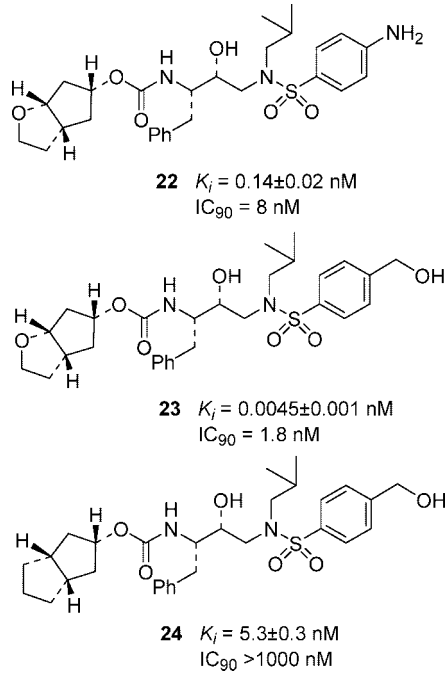


FIGURE 7. Structures of Inhibitors **22**–**24**.

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

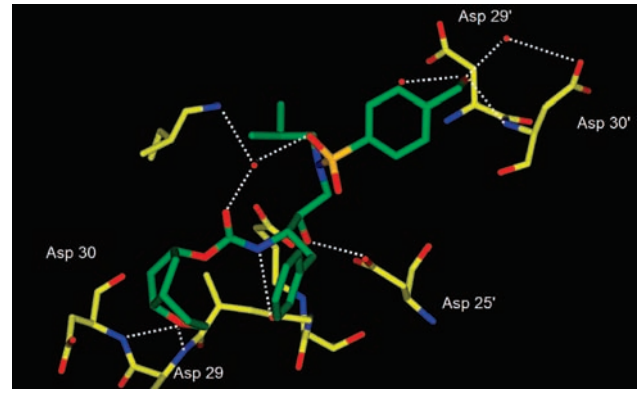


FIGURE 8. Inhibitor **23**-bound X-ray structure of HIV-1 protease.

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

Bis-THF-Derived Novel PIs

We have further explored a number of P_2' sulfonamide functionalities to interact with the backbone atoms in the S_2' subsite. As shown in Table 3, inhibitors **19**–**21** displayed exceedingly potent inhibitory properties. Inhibitor **21**, which contains a benzodioxolanesulfonamide derivative as its P_2' ligand, provided impressive enzyme inhibitory (<5 pM) and antiviral potency.[39] The antiviral activity of the inhibitors was evaluated against wild-type clinical isolates HIV-1_{LAI} and HIV-$1_{Ba\text{-}L}$ 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

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

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

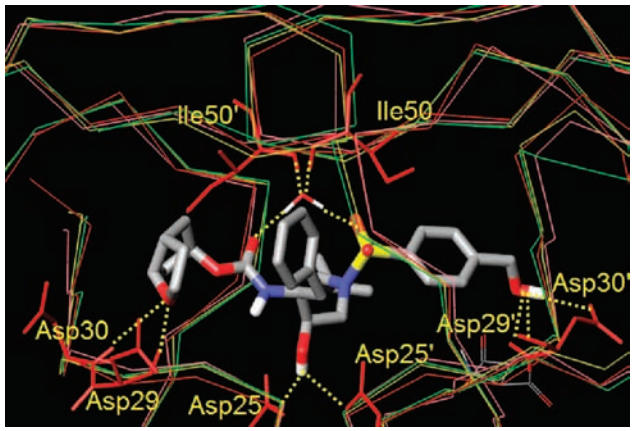


FIGURE 9. Inhibitor **23**-bound to the active site of wild-type HIV-1 protease superimposed upon the three most highly mutated drug-resistant proteases.

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

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

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

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

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

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

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

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

Conclusion

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

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

BIOGRAPHICAL INFORMATION

Arun K. Ghosh received his Ph.D. from the University of Pittsburgh and pursued postdoctoral research with Professor E. J. Corey at Harvard University. He was a Professor of Chemistry at the University of Illinois at Chicago from 1994 to 2005. From 2005 to present, he is a Professor in the departments of chemistry and medicinal chemistry at Purdue University.

Bruno D. Chapsal obtained his M.S. in chemistry from CPE Lyon, France, and received his Ph.D. from Stony Brook University, NY, under the direction of Professor Iwao Ojima. He is currently carrying out postdoctoral research in Professor Ghosh's laboratories.

Irene T. Weber received her Ph.D. from the University of Oxford, England, under the supervision of Professor Louise Johnson. She pursued postdoctoral research with Professor Thomas Steitz at Yale University. She was Professor of Microbiology and Immunology at Thomas Jefferson University in Philadelphia from 1991 to 2000. From 2001 to the present she is Professor of Biology and Chemistry at Georgia State University in Atlanta.

Hiroaki Mitsuya received his M.D. and Ph.D. from the Kumamoto University School of Medicine, Japan. He was a Visiting Scientist at the National Cancer Institute from 1982 to 1990. From 2001 to present, he is Principal Investigator & Chief, Experimental Retrovirology Section, HIV and AIDS Malignancy Branch, National Cancer Institute, Bethesda, MD. From 1997 to present, he is also Professor of Medicine and Chairman, Department of Internal Medicine, Kumamoto University School of Medicine, Japan.

FOOTNOTES

*To whom correspondence should be addressed. Fax: 765-496-1612. E-mail: akghosh@purdue.edu.

REFERENCES

1 Barre-Sinoussi, F.; Chermann, J. C.; Rey, F.; Nugeyre, M. T.; Chamaret, S.; Gruest, J.; Dauguet, C.; Axler-Blin, C.; Vezinet-Brun, F.; Rouzioux, C.; Rozenbaum, W.; Montagnier, L. Isolation of a T-lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS). *Science* **1983**, *220*, 868–871.

2 Gallo, R. C.; Salahuddin, S. Z.; Popovic, M.; Shearer, G. M.; Kaplan, M.; Haynes, B. F.; Palker, T. J.; Redfield, R.; Oleske, J.; Safai, B.; White, G.; Foster, P.; Markham, P. D. Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and at Risk for AIDS. *Science* **1984**, *224*, 500–503.

3 The impact of AIDS on People and Societies/2006, report on the Global AIDS Epidemic, http://data.unaids.org/pub/GlobalReport/2006/2006_GR_CH04_en.pdf

4 De Clercq, E. New Approaches toward Anti-HIV Chemotherapy. *J. Med. Chem.* **2005**, *48*, 1297–1313.

5 Graves, M. C.; Lim, J. J.; Heimer, E. P.; Kramer, R. A. An 11-kDa Form of Human Immunodeficiency Virus Protease Expressed in Escherichia Coli is Sufficient for Enzymatic Activity. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 2449–2453.

6 Wlodawer, A.; Vondrasek, J. Inhibitors of HIV-1 Protease: A Major Success of Structure-Assisted Drug Design. *Annu. Rev. Biophys. Biomol. Struct.* **1998**, *27*, 249–284.

7 The First HIV Protease Inhibitors Approved by FDA. *Antiviral Agents Bull.* **1995**, *8*, 353–355.

8 Sepkowitz, K. A. AIDS—The First 20 Years. *N. Engl. J. Med.* **2001**, *344*, 1764–1772.

9 Grabar, S.; Pradier, C.; Le Corfec, E.; Lancar, R.; Allavena, C.; Bentata, M.; Berlureau, P.; Dupont, C.; Fabbro-Peray, P.; Poizot-Martin, I.; Costagliola, D. Factors Associated with Clinical and Virological Failure in Patients Receiving a Triple Therapy Including a Protease Inhibitor. *AIDS* **2000**, *14*, 141–149.

10 Wainberg, M. A.; Friedland, G. Public Health Implications of Antiretroviral Therapy and HIV Drug Resistance. *J. Am. Med. Assoc.* **1998**, *279*, 1977–1983.

11 Hong, L.; Zhang, X.; Hartsuck, J. A.; Tang, J. Crystal Structure of an In Vivo HIV-1 Protease Mutant in Complex with Saquinavir: Insights into the Mechanisms of Drug Resistance. *Protein Sci.* **2000**, *9*, 1898–1904.

12 Laco, G. S.; Schalk-Hihi, C.; Lubkowski, J.; Morris, G.; Zdanov, A.; Olson, A.; Elder, J. H.; Wlodawer, A.; Gustchina, A. Crystal Structures of the Inactive D30N Mutant of Feline Immunodeficiency Virus Protease Complexed with a Substrate and an Inhibitor. *Biochemistry* **1997**, *36*, 10696–10708.

13 Glesby, M. J. Toxicities and Adverse Effects of Protease Inhibitors. In *Protease Inhibitors in AIDS Therapy*; Ogden, R. C., Flexner, C. W., Eds.; Marcel Dekker: New York, 2001; pp 237–256.

14 Krohn, A.; Redshaw, S.; Ritchie, J. C.; Graves, B. J.; Hatada, M. H. Novel Binding Mode of Highly Potent HIV-Proteinase Inhibitors Incorporating the (R)-Hydroxyethylamine Isostere. *J. Med. Chem.* **1991**, *34*, 3340–3342.

15 Ghosh, A. K.; Kincaid, J. F.; Walters, D. E.; Chen, Y.; Chaudhuri, N. C.; Thompson, W. J.; Culberson, C.; Fitzgerald, P. M. D.; Lee, H. Y.; McKee, S. P.; Munson, P. M.; Duong, T. T.; Darke, P. L.; Zugay, J. A.; Schleif, W. A.; Axel, M. G.; Lin, J.; Huff, J. R. Nonpeptidal P_2 Ligands for HIV Protease Inhibitors: Structure-Based Design, Synthesis, and Biological Evaluation. *J. Med. Chem.* **1996**, *39*, 3278–3290.

16 Ghosh, A. K.; Thompson, W. J.; McKee, S. P.; Duong, T. T.; Lyle, T. A.; Chen, J. C.; Darke, P. L.; Zugay, J. A.; Emini, E. A.; Schleif, W. A.; Huff, J. R.; Anderson, P. S. 3-Tetrahydrofuran and Pyran Urethanes as High-Affinity P_2-Ligands for HIV-1 Protease Inhibitors. *J. Med. Chem.* **1993**, *36*, 292–294.

17 Kim, E. E.; Baker, C. T.; Dwyer, M. D.; Murcko, M. A.; Rao, B. G.; Tung, R. D.; Navia, M. A. Crystal Structure of HIV-1 Protease in Complex with VX-478, a Potent and Orally Bioavailable Inhibitor of the Enzyme. *J. Am. Chem. Soc.* **1995**, *117*, 1181–1182.

18 Ghosh, A. K.; Thompson, W. J.; Fitzgerald, P. M. D.; Culberson, J. C.; Axel, M. G.; McKee, S. P.; Huff, J. R.; Anderson, P. S. Structure-based Design of HIV-1 Protease Inhibitors: Replacement of two Amides and a 10π-Aromatic System by a Fused Bis-Tetrahydrofuran. *J. Med. Chem.* **1994**, *37*, 2506–2508.

19 Nakanishi, K. The Ginkgolides. *Pure Appl. Chem.* **1967**, *14*, 89–114.

20 Corey, E. J.; Kang, M. C.; Desai, M. C.; Ghosh, A. K.; Houpis, I. N. Total Synthesis of (+/−)-Ginkgolide-B. *J. Am. Chem. Soc.* **1988**, *110*, 649–651.

21 Corey, E. J.; Ghosh, A. K. Total Synthesis of Ginkgolide A. *Tetrahedron Lett.* **1988**, *29*, 3205–3206.

22 Ghosh, A. K.; Chen, Y. Synthesis and Optical Resolution of High Affinity P_2 Ligands for HIV-1 Protease Inhibitors. *Tetrahedron Lett.* **1995**, *36*, 505–508.

23 Ghosh, A. K.; Leschenko, S.; Noetzel, M. Stereoselective Photochemical 1,3-Dioxolane Addition to 5-Alkoxymethyl-2(5*H*)-furanone: Synthesis of Bis-Tetrahydrofuranyl Ligand for HIV Protease Inhibitor UIC-94017 (TMC-114). *J. Org. Chem.* **2004**, *69*, 7822–7829.

24 Ghosh, A. K.; Li, J.; Sridhar, P. R. A Stereoselective Anti-Aldol Route to (3*R*,3a*S*,6a*R*)-Hexahydrofuro[2,3-*b*]furan-3-ol: A Key Ligand for a New Generation of HIV Protease Inhibitors. *Synthesis* **2006**, *18*, 3015–3018.

25 Ghosh, A. K.; Kincaid, J. F.; Cho, W.; Walters, D. E.; Krishnan, K.; Hussain, K. A.; Koo, Y.; Cho, H.; Rudall, C.; Holland, L.; Buthod, J. Potent HIV Protease Inhibitors Incorporating High-Affinity P_2-Ligands and (*R*)-(Hydroxyethylamino)sulfonamide Isostere. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 687–690.

26 Ghosh, A. K.; Pretzer, E.; Cho, H.; Hussain, K. A.; Duzgunes, N. Antiviral Activity of UIC-PI, a Novel Inhibitor of the Human Immunodeficiency Virus Type 1 Protease. *Antiviral Res.* **2002**, *54*, 29–36.

27 Yoshimura, K.; Kato, R.; Kavlick, M. F.; Nguyen, A.; Maroun, V.; Maeda, K.; Hussain, K. A.; Ghosh, A. K.; Gulnik, S. V.; Erickson, J. W.; Mitsuya, H. A Potent Human Immunodeficiency Virus Type 1 Protease Inhibitor, UIC-94003 (TMC-126), and Selection of a Novel (A28S) Mutation in the Protease Active Site. *J. Virol.* **2002**, *76*, 1349–1358.

28 Koh, Y.; Nakata, H.; Maeda, K.; Ogata, H.; Bilcer, G.; Devasamudram, T.; Kincaid, J. F.; Boross, P.; Wang, Y.-F.; Tie, Y.; Volarath, P.; Gaddis, L.; Harrison, R. W.; Weber, I. T.; Ghosh, A. K.; Mitsuya, H. Novel *bis*-Tetrahydrofuranylurethane-Containing Nonpeptidic Protease Inhibitor (PI) UIC-94017 (TMC114) with Potent Activity against Multi-PI-Resistant Human Immunodeficiency Virus In Vitro. *Antimicrob. Agents Chemother.* **2003**, *47*, 3123–3129.

29 De Meyer, S.; Azijn, H.; Surleraux, D.; Jochmans, D.; Tahri, A.; Pauwels, R.; Wigerinck, P.; de Bethune, M.-P. TMC114, a Novel Human Immunodeficiency Virus Type 1 Protease Inhibitor Active against Protease Inhibitor-Resistant Viruses, Including a Broad Range of Clinical Isolates. *Antimicrob. Agents Chemother.* **2005**, *49*, 2314–2321.

30 Tie, Y.; Boross, P. I.; Wang, Y.-F.; Gaddis, L.; Hussain, A. K.; Leshchenko, S.; Ghosh, A. K.; Louis, J. M.; Harrison, R. W.; Weber, I. T. High Resolution Crystal Structures of HIV-1 Protease with a Potent Non-Peptide Inhibitor (UIC-94017) Active against Multi-Drug-Resistant Clinical Strains. *J. Mol. Biol.* **2004**, *338*, 341–352.

31 Kovalevsky, A. Y.; Tie, Y.; Liu, F.; Boross, P. I.; Wang, Y.-F.; Leshchenko, S.; Ghosh, A. K.; Harrison, R. W.; Weber, I. T. Effectiveness of Nonpeptide Clinical Inhibitor TMC-114 on HIV-1 Protease with Highly Drug Resistant Mutations D30N, I50V, and L90M. *J. Med. Chem.* **2006**, *49*, 1379–1387.

32 Kovalevsky, A. Y.; Liu, F.; Leshchenko, S.; Ghosh, A. K.; Louis, J. M.; Harrison, R. W.; Weber, I. T. Ultra-High Resolution Crystal Structure of HIV-1 Protease Mutant Reveals Two Binding Sites for Clinical Inhibitor TMC114. *J. Mol. Biol.* **2006**, *363*, 161–173.

33 King, N. M.; Prabu-Jeyabalan, M.; Nalivaika, E. A.; Wigerinck, P.; de Bethune, M.-P.; Schiffer, C. A. Structural and Thermodynamic Basis for the Binding of TMC114 a Next-Generation Human Immunodeficiency Virus Type 1 Protease Inhibitor. *J. Virol.* **2004**, *78*, 12012–12021.

34 De Meyer, S.; Peters, M. Abstracts 533 and 620, 11th Conference on Retroviruses and Opportunistic Infections (CROI); Feb 8–11 2004, San Francisco, CA.

35 Hoetelmans, R.; van der Sandt, I.; De Pauw, M.; Struble, K.; Peeters, M.; van der Geest, R. TMC114, a Next Generation HIV Protease Inhibitor: Pharmacokinetics and Safety Following Oral Administration of Multiple Doses with or without Low Doses of Ritonavir in Healthy Volunteers; Abstract 549, 10th Conference on Retroviruses and Opportunistic Infections (CROI); Feb 2003, Boston, MA.

36 Katlama, C.; Carvalho, M. T. M.; Cooper, D.; De Backer, K.; Lefebvre, E.; Pedro, R.; Rombouts, K.; Stoehr, A.; Vangeneugden, T.; Woehrmann, A. TMC114/r Outperforms Investigators-selected PI(s) in 3-class-experienced Patients: Week 24 Efficacy Analysis of POWER 1 (TMC114-C213) [Poster WeOalB0102], 3rd IAS Conference on HIV Pathogenesis and Treatment; July 24–27 2005, Rio de Janeiro, Brazil.

37 Clotet, B.; Bellos, N.; Molina, J.-M.; Cooper, D.; Goffard, J.-C.; Lazzarin, A.; Wohrmann, A.; Katlama, C.; Wilkin, T.; Haubrich, R.; Cohen, C.; Farthing, C.; Jayaweera, D.; Markowitz, M.; Ruane, P.; Spinosa-Guzman, S.; Lefebvre, E. Efficacy and Safety of Darunavir-Ritonavir at Week 48 in Treatment-experienced Patients with HIV-1 Infection in POWER 1 and 2: a Pooled Subgroup Analysis of Data from Two Randomised Trials. *Lancet* **2007**, *369*, 1169–1178.

38 FDA approves Darunavir on June 23, 2006: FDA approved new HIV treament for patients who do not respond to existing drugs. Please see http://www.fda.gov/bbs/topics/NEWS/2006/NEW01395.html

39 Amano, M.; Koh, Y.; Das, D.; Li, J.; Leschenko, S.; Wang, Y.-F.; Boross, P. I.; Weber, I. T.; Ghosh, A. K.; Mitsuya, H. A Novel *Bis*-Tetrahydrofuranylurethane-Containing Nonpeptidic Protease Inhibitor (PI), GRL-98065, Is Potent against Multi-PI-Resistant Human Immunodeficiency Virus *In Vitro*. *Antimicrob. Agents Chemother.* **2007**, *51*, 2143–2155.

40 Spaltenstein, A.; Kazmierski, W. M.; Miller, J. F.; Samano, V. Discovery of Next Generation Inhibitors of HIV Protease. *Curr. Top. Med. Chem.* **2005**, *5*, 1589–1607.

41 Ghosh, A. K.; Sridhar, P. R.; Leshchenko, S.; Hussain, A. K.; Li, J.; Kovalevsky, A. Y.; Walters, D. E.; Wedekind, J. E.; Grum-Tokars, V.; Das, D.; Koh, Y.; Maeda, K.; Gatanaga, H.; Weber, I. T.; Mitsuya, H. Structure-based Design of Novel HIV-1 Protease Inhibitors to Combat Drug Resistance. *J. Med. Chem.* **2006**, *49*, 5252–5261.

42 Miller, J. F.; Andrews, C. W.; Brieger, M.; Furfine, E. S.; Hale, M. R.; Hanlon, M. H.; Hazen, R. J.; Kaldor, I.; McLean, E. W.; Reynolds, D.; Sammond, D. M.; Spaltenstein, A.; Tung, R.; Turner, E. M.; Xu, R. X.; Sherrill, R. G. Ultra-Potent P_1 Modified Arylsulfonamide HIV Protease Inhibitors: The Discovery of GW0385. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1788–1794.

43 Clemente, J. C.; Moose, R. E.; Hemrajani, R.; Whitford, L. R.; Govindasamy, L.; Reutzel, R.; McKenna, R.; Agbandje-McKenna, M.; Goodenow, M. M.; Dunn, B. M. Comparing the Accumulation of Active- and Nonactive-site Mutations in the HIV-1 Protease. *Biochemistry* **2004**, *43*, 12141–12151.

44 Chen, Z.; Li, Y.; Chen, E.; Hall, D. L.; Darke, P. L.; Culberson, C.; Shafer, J. A.; Kuo, L. C. Crystal Structure at 1.9-Å Resolution of Human Immunodeficiency Virus (HIV) II Protease Complexed with L-735,524, an Orally Bioavailable Inhibitor of the HIV Proteases. *J. Biol. Chem.* **1994**, *269*, 26344–26348.