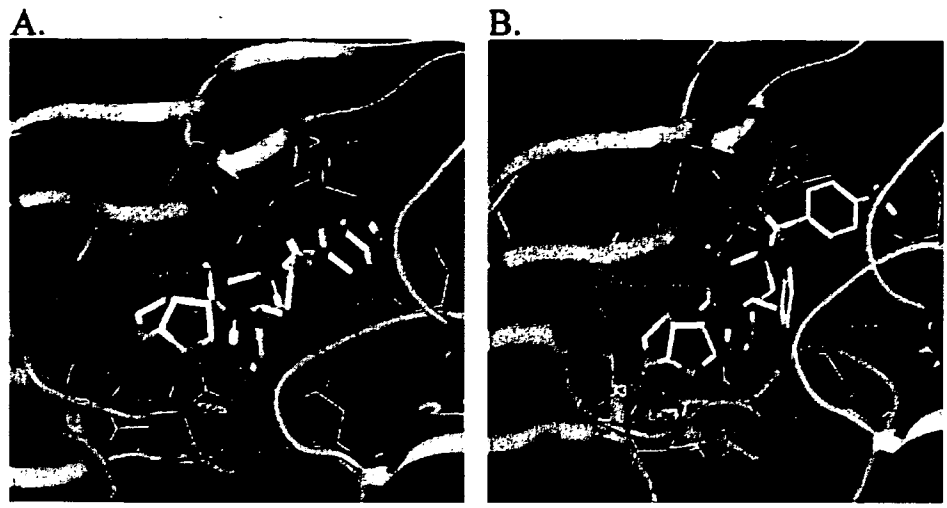

FIG. 7. Selected hydrogen bond interactions of GRL-98065 with wild-type HIV-1 protease. (A) The bis-THF group forms hydrogen bond interactions with backbone atoms of Asp29 and Asp30. There is a hydrogen bond with the backbone atom of Gly27. The hydroxyl group forms hydrogen bonds with the side chains of the catalytic aspartates. One oxygen of the benzodioxole group forms a hydrogen bond interaction with Asp30′, and the other oxygen of the benzodioxole group forms a water-mediated hydrogen bond interaction with Gly48′. (B) Hydrogen bond interactions between DRV and protease (PDB identifier, 1S6G) are shown. Most interactions between GRL-98065 and DRV are similar, except for interactions with Asp30′ and Gly48′. GRL-98065 interacts with the Asp30′ amide, while DRV interacts with the Asp30′ carbonyl oxygen. The benzodioxole oxygen of GRL-98065 has a water-mediated interaction with Gly48′ in the flap. This interaction appears to stabilize the binding site more for GRL-98065 and may be partly responsible for its greater antiviral potency than that of DRV.

docking calculations show that for these mutant proteases, GRL-98065 is able to maintain most of the four hydrogen bond interactions observed for the wild-type protease. In particular, we observed that the hydrogen bond interaction with Asp29 is maintained in four out of six mutant proteases. Taken together, these results are likely to explain why GRL-98065 is able to show greater potency than other clinically approved PIs against a wide spectrum of multiple-PI-resistant HIV-1 variants.

TABLE 5. Hydrogen bond distances of protease inhibitors with selected active-site residues[a]

Inhibitor	PDB ID	Hydrogen bond distance(s) (Å)		
		Asp29	Asp30	Asp30′
GRL-98065		1.9/2.4	2.4	2.5
DRV	1S6G	2.3/2.4	2.4	2.5
SQV	1HXB	1.9	2.2	NP
RTV	1HXW	2.1	NP	2.2
IDV	1SDT	2.1	NP	NP
NFV	1OHR	NP	NP	NP
APV	1HPV	2.8	2.6	NP
LPV	1MUI	1.7	NP	NP
ATV	2AQU	1.9	NP	NP

[a] Hydrogen atoms were added and optimized with constraints on heavy atoms using the OPLS2005 force field (MacroModel, version 9.1; Schrödinger, LLC). Hydrogen bond tolerances used were as follows: 3.0 Å for H—A distance; D—H—A angle greater than 90°; and H—A—B angle greater than 60°, where H is the hydrogen atom, A is the acceptor, D is the donor, and B is a neighbor atom bonded to the acceptor. Values for separate interactions are separated by a shill. NP (not present) denotes that a hydrogen bond is not present between the inhibitor and the particular residue. ID, identifier.

DISCUSSION

GRL-98065, which contains a unique component, bis-THF, and a sulfonamide isostere, suppressed a wide spectrum of HIV-1, HIV-2, and primary HIV-1 strains of different subtypes over a very narrow spread of EC_{50}s ranging from 0.0002 to 0.0045 μM (Tables 1 and 3). GRL-98065 was highly potent against a variety of multidrug-resistant clinical HIV-1 isolates, with EC_{50}s of 0.003 to 0.006 μM, while the existing FDA-approved PIs examined either failed to suppress the replication of those isolates or required much higher concentrations for viral inhibition (Table 3). When examined against laboratory PI-selected HIV-1 variants (except against HIV-$1_{APV5\mu M}$), GRL-98065 also exerted potent activity, with EC_{50}s ranging 0.0015 to 0.0075 μM (Table 2). It is of note that GRL-98065 was less potent against APV-resistant HIV-$1_{APV5\mu M}$; however, it is thought that this relative cross-resistance is due to the structural similarities of GRL-98065 with APV. It is intriguing that the activity of SQV against laboratory PI-selected variants, except for the SQV-selected variant, was fairly well maintained, a profile generally consistent with our results and those of other groups (11, 32, 33). However, when SQV was examined against multidrug-resistant primary HIV-1 strains, high concentrations of SQV were required to suppress the replication of four of six strains tested (EC_{50}s ranging from 0.14 to 0.29 μM) (Table 3). In contrast, GRL-98065 exerted highly potent activity against all the six primary strains examined. As shown in Table 3, even the replication of the most PI-resistant primary strain, HIV-1_{JSL}, against which EC_{50}s of the eight PIs, including ATV and DRV, were 0.027 to >1 μM, was effectively suppressed by GRL-98065 at a fairly low concentration, with an EC_{50} of 0.006 μM.

FIG. 8. Interactions between GRL-98065 and wild-type protease. van der Waals surfaces of GRL-98065 (green), Val82 (red), and Ile85 (magenta) are shown. There are strong van der Waals interactions of GRL-98065 with Val82 and Val82'. Note that Val82 was replaced with isoleucine as a primary resistance mutation during in vitro passage of HIV-1 in the presence of GRL-98065. However, Ile85 does not have van der Waals contact with the inhibitor, suggesting that I85V emerged as a secondary mutation during in vitro selection with the inhibitor.

The observed greater potency of GRL-98065 than those of the existing FDA-approved PIs examined in the present study appears to stem, at least in part, from the ability of the two conformationally constrained ring oxygen atoms in its bis-THF group to form hydrogen bonds with the main chain amide hydrogen atoms of Asp29 and Asp30 in the S2 subsite (Fig. 7). Since the main chain atoms cannot be changed by viral amino acid substitutions, the interactions of GRL-98065 and the two catalytic site amino acids are unlikely to be substantially affected, perhaps resulting in GRL-98065's broad spectrum of activity against multidrug-resistant variants.

It is noteworthy that Asp30 can be mutated to asparagine when HIV-1 is exposed to NFV (26). This mutation, D30N, is a primary resistance mutation for NFV that results in formation of a hydrogen bond with the side chain of Asp30 (26). GRL-98065 does not have direct interaction with the side chain of Asp30 (Fig. 7). Consistent with this observation, exposure of HIV-1 to GRL-98065 did not select mutations at codon 30, and GRL-98065 was active against D30N-carrying HIV-$1_{NFV5\mu M}$, which was highly resistant to NFV, with an EC_{50} value of >1 μM (Table 2).

In the present HIV-1 selection experiment with GRL-98065, by passage 30 and beyond, 10 major amino acid substitutions (E21K, A28S, K43I, M46I, I50V, D60N, A71V, V82I, I85V, and L89M) were identified. It is noteworthy that mutation of Val82, whose side chain makes direct contacts with a number of PIs (3), was not seen in HIV-1 selected with TMC126 that has bis-THF and exerts potent activity against a wide spectrum of HIV-1 strains (32). Presumably, V82I arises due to the fact that GRL-98065 has a tight and direct contact with Val82 (Fig. 8), while Ile85 does not have van der Waals contact with the inhibitor, suggesting that I85V emerged as a secondary mutation during the in vitro selection with GRL-98065 (Fig. 4). In GRL-98065-selected HIV-1, neither of the active-site amino acid substitutions, I84V or V32I, emerged. These two substitutions are known to confer high levels of PI resistance on HIV-1, in particular when combined with V82I (17). The absence of these mutations may contribute to the observed delayed acquisition and relatively low level of resistance to GRL-98065.

It is also of note that during selection with GRL-98065, the unique A28S mutation in the active site of the enzyme emerged. The A28S mutation was seen during the selection of HIV-1_{NL4-3} with TMC126, where the mutation never became predominant but persisted within TMC126-selected HIV-1 variants at frequencies of ~50%. In a previous biochemical study conducted by Hong et al. (14), the A28S mutation in HIV protease caused a more than 1,500-fold decrease in k_{cat}/K_m values for peptide substrates. These results suggest that A28S represents a critical mutation for GRL-98065 resistance but also confers a severe replication disadvantage on the virus. It should be noted, however, that the population size of HIV-1 in a culture is relatively small and the appearance of mutations can be affected by stochastic phenomena, i.e., rates and orders of appearance of mutations. In order to address the issue of mutation appearance, clinical studies on GRL-98065 are ultimately needed.

The crystal structure reveals that GRL-98065 has a series of hydrogen bond interactions with backbone atoms of Asp29, Asp30, Asp30', and Gly27 of the protease (Fig. 7). Like other protease inhibitors, GRL98065 also has hydrogen bond interactions with the side chain atoms of Asp25 and Asp25'. Besides the water-mediated hydrogen bond interactions with Ile50 and Ile50', there is a water-mediated hydrogen bond interaction with the flap residue Gly48'. Thus, GRL-98065 makes favorable polar interactions with Asp29 and Asp30 as well as with the flap residues. These hydrogen bond interactions, besides various favorable van der Waals contacts, are likely to be responsible for the strong binding of the inhibitor and its potent antiviral activity observed in the present work. Comparison of the crystal structure of HIV-1 protease with that of GRL-98065 and the crystal structure of the complex with the recently approved inhibitor DRV shows that the interactions with the S2 site of the protease are shared by the two PIs, but the nature of the hydrogen bonds with residues in the S2' site differs (Fig. 7). The water-mediated interaction of GRL-98065 with flap residue 48' is not observed for DRV. These differences in interactions might be partly responsible for the low EC_{50} of GRL-98065 compared to that of DRV (Tables 1 to 3).

We also attempted to gain a structural understanding of why GRL-98065 is able to maintain a highly favorable potency against a variety of laboratory PI-resistant HIV-1 variants and multidrug-resistant clinical isolates. The resistance of PIs due to mutations arises because of possible loss of direct hydrogen bond interactions with specific residues (e.g., D30N for NFV and G48V for SQV) or loss of van der Waals contact (e.g., with V82A and I84V for first-generation PIs). Analysis of mutant protease crystal structures in comparison with that of wild-type protease showed that the backbone atoms of mutant protease undergo minimal conformational changes on mutation (9, 15, 19). The loss of binding in many cases seems to be due to loss of weaker van der Waals contacts between the inhibitor and the protease. We hypothesize

that if an inhibitor maintains strong hydrogen bond interactions with the wild-type protease, particularly with backbone atoms of multiple residues that are conserved (e.g., Asp29 and Gly27), then the loss of van der Waals contacts due to mutations may not result in a drastic loss of binding affinity. Thus, inhibitors without multiple strong hydrogen bond interactions with wild-type protease would be more susceptible to loss of binding due to loss of weaker van der Waals contacts than inhibitors with multiple hydrogen bond interactions. In this respect, we analyzed the hydrogen bond interactions of several PIs with wild-type protease (Table 5). It is noteworthy that only GRL-98065 and DRV have four hydrogen bond interactions with backbone atoms of Asp29 and Asp30 and of Asp30′. None of the other clinically approved PIs studied here have more than two hydrogen bond interactions with these residues. Thus, GRL-98065 is likely to preserve the hydrogen bond interactions and bind tightly with mutant protease.

The present data suggest that GRL-98065 has several advantages: (i) it exerts potent activity against a wide spectrum of drug-resistant HIV-1 variants, presumably due to its interactions with the main chains of the active-site amino acids Asp29 and Asp30; (ii) its unique contact with HIV-1 protease differs from that of other PIs; (iii) the viral acquisition of resistance is substantially delayed; and (iv) at least several PIs, including SQV and ATV, remain active in vitro against the virus selected in vitro with GRL-98065. It is of note that GRL-98065 possesses substantially favorable features as a potential therapeutic for AIDS, as described above; however, its oral bioavailability, pharmacokinetics/pharmacodynamics, biodistribution, etc., are yet to be determined in further rigorous preclinical and clinical testing.

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Spirodiketopiperazine-based CCR5 antagonists: Lead optimization from biologically active metabolite

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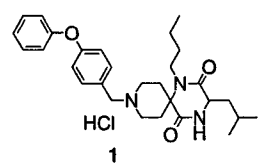
Abstract—Hydroxylated derivatives were designed and synthesized based on the information of oxidative metabolites. Compounds derived from β-substituted (2*R*,3*R*)-2-amino-3-hydroxypropionic acid showed improved inhibitory activities against the binding of MIP-1α to human CCR5, compared with the non-hydroxylated derivatives and the other isomers.

Millions of people in the world are still suffering from acquired immune deficiency syndrome (AIDS).[1] Although the highly active antiretroviral therapy (HAART), a cocktail of protease and reverse transcriptase inhibitors, has been useful for many patients, several issues still remain for anti-HIV therapy: a gradual spread of drug-resistant strains, severe adverse effects, expensive therapeutic cost, etc.[2] These issues require new anti-HIV drugs to have a different mode of action from conventional drugs.

Agents inhibiting HIV entry into target cells are one of the most promising approaches to treat AIDS.[3] A number of potential sites for therapeutic intervention become accessible during the narrow window between virus attachment and the subsequent fusion of viral envelope with the cell membrane. In 1996, it was revealed that one of the C–C chemokine receptor 5 (CCR5) is utilized by HIV-1 as an essential co-receptor and that the endogenous ligand showed anti-HIV-1 activity in vitro.[4] CCR5 belongs to the superfamily of G protein-coupled receptors (GPCRs), which greater than 30% of all known marketed medicines modulate the function of.[5] After these reports, many pharmaceutical companies and academic institutions have been enthusiastically investigating novel antagonists against CCR5 with suitable pharmaceutical properties.[6]

We previously reported the identification of several spirodiketopiperazine derivatives, for example, **1** (Fig. 1), from a combinatorial library targeting chemokine receptors.[7] Compound **1** not only selectively inhibited the binding of macrophage inflammatory protein (MIP)-1α to human CCR5 receptor, but also potently blocked the infectivity and replication of laboratory and clinical strains of HIV as well as those of highly drug-resistant HIV variants with minimal cytotoxicity.[8] Although



1

Ca Assay IC_{50} = 94 nM
Binding Assay IC_{50} = 8.3 nM
Anti-HIV Activity IC_{90} = 676 nM
Rat B.A.= 1.3%

Figure 1. The structure of lead compound **1**.

Keywords: CCR5; HIV-1; Active metabolite.
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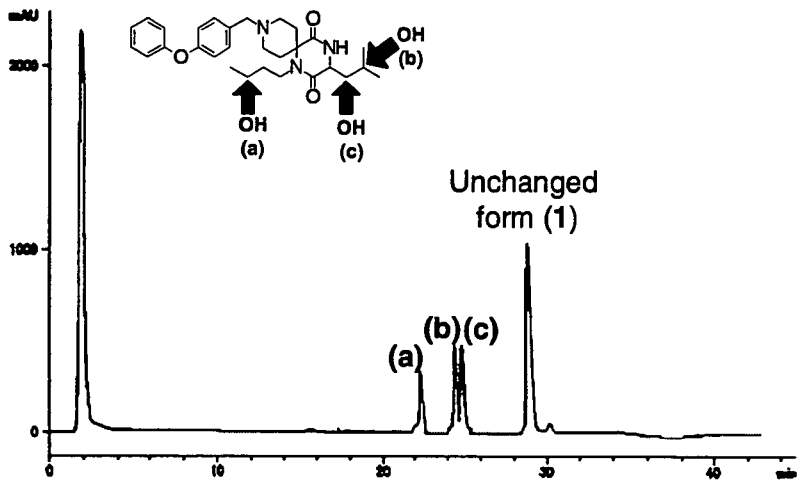


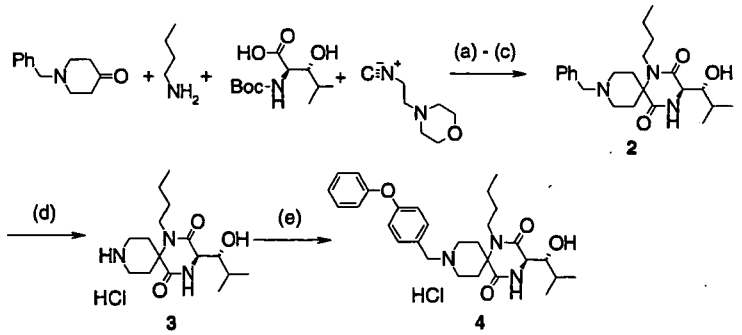
Figure 2. Metabolites of the compound **1**. Three main metabolites were identified on LC-MS after incubation with human liver microsome (5.0 mg protein/ml) for 1.5 h.

compound **1** showed potent activity in vitro, oxidative metabolism in liver microsomes resulted in low bioavailability in rodents.

After the incubation with human liver microsomes, metabolites of **1** were purified by HPLC. The three major isolated metabolites (a, b, c in Fig. 2) were analyzed by LC-MS and ^{1}H NMR, and found to be compounds hydroxylated on the *n*-butyl and/or the *i*-butyl group.[9] Additionally, we evaluated the in vitro antagonistic activity of each isolated metabolite. Fortunately, the samples of the peaks (b) and (c) showed significant antagonistic activities (data not shown). This information prompted us to try the introduction of hydroxyl group on side chains to improve the in vitro activity as well as pharmaceutical properties. Herein, we describe the preliminary structure–activity relationship (SAR) of the hydroxylated-spirodiketopiperazines and the unexpected improvement on the activities, especially in vitro anti-HIV activities.

The compounds **1**, **5**, **7**, **8**, and **12** were synthesized from the *N*-alloc-4-piperidone, the corresponding amine, the corresponding *N*-Boc-amino acid, and 4-phenoxybenzaldehyde by the reported solid-phase synthesis.[7] The compounds **9–11** and **13** were synthesized from the corresponding amino acid derivatives by the identical procedure to the synthesis of compound **4** shown in Scheme 1. The mixture of 1-benzylpiperidone, butylamine, *N*-Boc-β-hydroxy-D-leucine, and 2-(4-morpholinyl)ethylisocyanide[10] in methanol was stirred at 55 °C.[11] The enantiomerically pure β-hydroxy-α-amino acids were prepared according to the reported method through Sharpless asymmetric epoxidation from the corresponding allyl alcohol.[12] The Boc protecting group of amino acid was removed by the treatment of concentrated HCl without isolation of the Ugi product. Cyclization of the obtained crude product by heating in toluene in the presence of acetic acid at 80 °C followed by the removal of the benzyl group by catalytic hydrogenation afforded the cyclized spirodiketopiperazine, and compound **3** was isolated as a HCl salt in acceptable yield. Reductive alkylation of compound **3** resulted in desired product **4** in high yields.

The compounds listed in Tables 1–3 were evaluated for their inhibitory activities against calcium mobilization of human CCR5 overexpressed CHO cell (hCCR5/



Scheme 1. Typical synthetic route for spirodiketopiperazines. Reagents and condition: (a) MeOH, 55 °C; (b) concd HCl, 55 °C; (c) AcOH/toluene, 80 °C; (d) H_2, $Pd(OH)_2$/C, EtOH, 55 °C then 4 N HCl/AcOEt (60–70% in four steps); (e) 4-phenoxybenzaldehyde, $NaBH(OAc)_3$, AcOH, DMF then 4 N HCl/AcOEt (80%).

Table 1. Activity of the compounds **5** and **6**

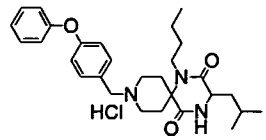
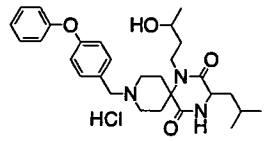
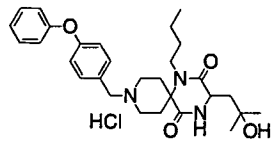
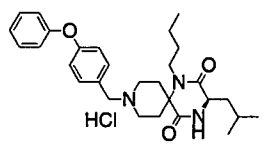
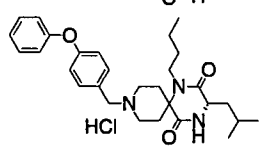
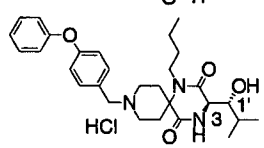
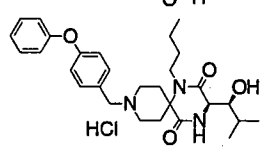
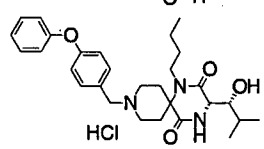
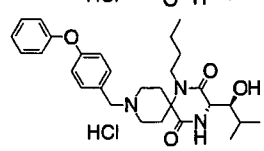
Compound	Structure	IC_{50}	
		Binding assay (nM)	Ca assay (nM)
1		8.3	94
5		Not tested	Ca. 10,000
6		28	79

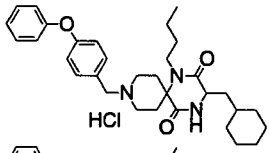
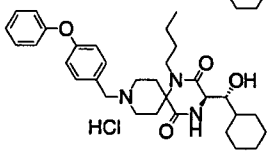
Table 2. Activity of the stereoisomers **4** and **7–11**

Compound	Structure	IC_{50}	
		Binding assay (nM)	Ca assay (nM)
7 *R* form		29	130
8 *S* form		11	84
4 (3*R*,1′*R*) form		3.5	33
9 (3*R*,1′*S*) form		24	210
10 (3*S*,1′*R*) form		68	400
11 (3*S*,1′*S*) form		53	150

CHO) stimulated by MIP-1α (Ca assay) and for their CCR5 binding affinity by the inhibition of ^{125}I-MIP-1α binding to hCCR5/CHO (binding assay).[8,13]

The compound **5**, the proposed structure of metabolite (a), was synthesized from the 3-hydroxy-1-butylamine and *N*-Boc-leucine as a mixture containing the same

Table 3. Antagonistic activity and anti-HIV activity in compounds **12** and **13**

Compound	Structure	Binding assay IC_{50} (nM)	Ca assay IC_{50} (nM)	Anti-HIV IC_{50} (nM)	MAGI assay IC_{50} (nM)
12		6.1	28	31	337
13		1.1	53	0.6	6.0

quantity of all possible four isomers. The compound **6**, the proposed structure of metabolite (b), was prepared from the *N*-Boc-aspartic acid derivative in racemic form.[14] Compounds **5** and **6** were evaluated for their activities and the results are summarized in Table 1. Whereas the compound **5** showed significant decrease of activity in calcium mobilization assay, the compound **6** showed a comparable activity to the parent compound **1**.[15]

Since the proposed structure of metabolite (c) had two chiral centers, we synthesized all four possible enantiomers **4** and **9–11** in optically pure form from the corresponding β-hydroxylated-leucine to evaluate their biological activities (Table 2). Whereas there was no remarkable difference between the activities of the two enantiomers, **7** and **8**, in lead compound **1**, there was a significant difference among the hydroxylated stereoisomers (**4** versus **9–11**). Compound **4** having 3*R*,1′*R*-configuration exhibited approximately 10-fold more potency than the other isomers **9–11**. This result indicates that introducing hydroxyl group on the side chain at the 3-position of diketopiperazine ring could lead to a significant improvement in the interaction with the receptor. Based on the observation of this unexpectedly improved antagonistic activity of compound **4**, we applied this information to compound **12** which showed more potent anti-HIV activity than compound **1**. It was found that the (3*R*,1′*R*)-hydroxyl compound **13** exhibited strong inhibitory activities in both binding and Ca assays. Furthermore, investigating anti-HIV activity in the next step, compound **13** showed 6 nM of IC_{90} value in anti-HIV assay ($CCR5^+$ MAGI cell anti-infectivity single cycle assay versus the BAL strain of HIV,[8a]), which was 50-fold stronger than non-hydroxyl analogue **12**.[16]

We also evaluated the oral bioavailability of compounds **4** and **13** in rat. Unfortunately, the bioavailability of both compounds was less than 1% (data not shown). Further assessment and optimization are required to identify promising clinical candidates with acceptable pharmaceutical profile.

In conclusion, using metabolite data of lead compound **1**, we discovered the excellent enhancement on the activity in binding and anti-HIV assays by the introduction of a β-hydroxyl group. Although the role of the hydroxyl group is still unclear, two hypotheses have been made to explain the increase in activity. One is the formation of a new hydrogen bond between the hydroxyl group and CCR5. The other is restricting the conformation of the compound to favorably orient the side chains. Further investigations are in progress.

Introduction of the hydroxyl group could not improve the bioavailability in rodent. However, the introduction of a hydrophilic moiety on the molecule showed some favorable pharmaceutical properties.[17] Further optimization of these compounds to improve their oral absorption and metabolic stability which are necessary to provide CCR5 antagonists suitable for clinical use will be discussed in our future reports.

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35194; (b) Maeda, K.; Nakata, H.; Koh, Y.; Miyakawa, T.; Ogata, H.; Takaoka, Y.; Shibayama, S.; Sagawa, K.; Fukushima, D.; Moravek, J.; Koyanagi, Y.; Mitsuya, H. *J. Virol.* **2004**, *78*, 8654.

9. ^{1}H NMR spectra of metabolites (a) and (c) indicated to be a single compound, respectively. However, it was difficult to determine the stereochemistry at each asymmetric center. As for the metabolite (c), the ^{1}H NMR data were identical to those of the compounds **4** and **11**, which indicates that this metabolite has *anti*-configuration.
10. We initially used several isocyanides, for example, benzylisocyanide, for this reaction to yield the desired product. However, most of isocyanides were difficult to be handled because of the offensive odor. It was found that a commercially available isocyanide, 2-(4-morpholinyl)ethylisocyanide, is not malodorous, and we decided to employ this reagent for the further derivatization.
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13. All IC_{50} values represent the average of two or more determinations, and the standard deviations were no greater than 30% from the mean.
14. Compound **6** was prepared by the reaction of methylmagnesium bromide with the benzyl ester derivative, which was obtained from the *N*-alloc-piperidone, butylamine, *N*-Boc-γ-benzyl-aspartic acid, and 4-phenoxybenzaldehyde according to the solid-phase synthesis reported in Ref. 7.
15. Compounds described herein showed selective CCR5 antagonistic activities over other chemokine receptors, for example, CCR1 and CXCR4.
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17. For example, whereas compound **12** inhibited CYP 3A4 at 9.3 μM of IC_{50}, compound **13** did not show any significant inhibition at 30 μM. Furthermore, compound **13** showed an improved aqueous solubility (pH 6.8) compared to compound **12** (**12**, <0.2 μg/ml; **13**, 2.7 μg/ml).

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Phenotypic and Genotypic Comparisons of Human T-Cell Leukemia Virus Type 1 Reverse Transcriptases from Infected T-Cell Lines and Patient Samples[▽]

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It is well established that cell-free infection with human T-cell leukemia virus type 1 (HTLV-1) is less efficient than that with other retroviruses, though the specific infectivities of only a limited number of HTLV-1 isolates have been quantified. Earlier work indicated that a postentry step in the infectious cycle accounted for the poor cell-free infectivity of HTLV-1. To determine whether variations in the *pol* gene sequence correlated with virus infectivity, we sequenced and phenotypically tested *pol* genes from a variety of HTLV-1 isolates derived from primary sources, transformed cell lines, and molecular clones. The *pol* genes and deduced amino acid sequences from 23 proviruses were sequenced and compared with 14 previously published sequences, revealing a limited number of amino acid variations among isolates. The variations appeared to be randomly dispersed among primary isolates and proviruses from cell lines and molecular clones. In addition, there was no correlation between reverse transcriptase sequence and the disease phenotype of the original source of the virus isolate. HTLV-1 *pol* gene fragments encoding reverse transcriptase were amplified from a variety of isolates and were subcloned into HTLV-1 vectors for both single-cycle infection and spreading-infection assays. Vectors carrying *pol* genes that matched the consensus sequence had the highest titers, and those with the largest number of variations from the consensus had the lowest titers. The molecular clone from CS-1 cells had four amino acid differences from the consensus sequence and yielded infectious titers that were approximately eight times lower than those of vectors encoding a consensus reverse transcriptase.

Human T-cell leukemia virus type 1 (HTLV-1) is an oncogenic retrovirus directly associated with adult T-cell leukemia (ATL) and HTLV-associated myelopathy/tropical spastic paraparesis. An estimated 10 to 20 million people worldwide are infected with the virus, with endemic foci in southern Japan, Melanesia, central Africa, the Caribbean, and South America (2, 31, 56). Like other retroviruses, HTLV-1 depends upon the activity of its reverse transcriptase (RT) for efficient replication in target cells. However, unlike other retroviruses, primate T-cell lymphotropic viruses and the related bovine leukemia virus are known for their low genetic diversity in vivo. Interpatient nucleotide sequence variability between isolates is less than 10% worldwide and usually less than 2% within the same geographic region, while intrapatient variability is considerably less (54). This extremely low genetic variability is likely due to provirus expansion by mitotic replication (60).

HTLV-1 does not productively infect established T-cell lines because of the cytostatic effects of Tax (32, 61). This is paradoxical, because it is through the action of Tax that HTLV-1 immortalizes primary T cells. Therefore, HTLV-1 isolates are generally obtained in the form of a provirus in a chronically infected cell line. HTLV-1-transformed cell lines are obtained by coculture of peripheral blood mononuclear cells (PBMC) or cord blood leukocytes with fresh peripheral blood leukocytes (PBLs) drawn from infected patients, producing mono- or oligoclonal cell lines. Although they typically harbor defective proviruses, these HTLV-1-transformed cell lines are capable of producing infectious virions. However, the cell-free virus particles released from these cell lines are poorly infectious. In the first quantitative study of cell-free HTLV-1 infection, it was estimated that only 1 in 10^6 virus particles from MT-2 cells were infectious (15).

The infectious HTLV-1 molecular clones pCS-HTLV and pACH were derived from the HTLV-transformed cell lines CS-1 and CH, respectively (9, 10, 26, 28, 52), and have been widely used to study HTLV-1 transformation and infectivity in vitro. The virus particles released by cells transfected with these provirus clones appear to mimic virions produced by transformed cell lines (9), but it is unclear whether the poor replication and infectivity of these viruses are typical of HTLV-1 in nature. We previously showed that pCS-HTLV-based vectors were about 1,000-fold less infectious than human immunodeficiency virus type 1 (HIV-1) when both viruses were pseudotyped with the same envelope (9). Thus, the difference in infectivity was at a postentry step, which likely reflected a difference in viral uncoating or reverse transcription. We have begun to characterize HTLV-1 RT to determine whether or not the RT contributes to the low infectious titer of cell-free HTLV-1 (33).

To determine whether a relationship exists between RT sequence variation and infectious titer, we sequenced and phe-

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notypically tested *pol* genes from a variety of sources in both single-cycle and spreading-infection assays. We found that isogenic molecular clones of HTLV-1 that contained the consensus RT gave titers that were eightfold higher than those of clones utilizing RT of the original molecular clone, pCS-HTLV.

MATERIALS AND METHODS

Cells and cell lines. Transfected human kidney (293T), human cervical carcinoma (HeLa), and fetal rhesus lung (FRhL-B5) (12) cells were maintained in Dulbecco's modified minimum essential medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics.

The following HTLV-1-transformed cell lines were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: C10/MJ (catalog no. 4407) from Dean Mann and Miklaus Popovic, MT-2 (catalog no. 237) and MT-4 (catalog no. 120) from Douglas Richman, and C8166 (catalog no. 404) from Robert Gallo (36, 37, 44, 48). Renu Lal and Charlene Dezzutti (Centers for Disease Control and Prevention, Atlanta, GA) generously provided the 1657, 3614, 3669, A212, EG, FS, and SP cell lines (7, 13, 16, 23, 47). The 1657, 3614, 3669, A212, C10/MJ, and C91/PL (44); C8166 and CS-1 (28); EG, FS, MT-2, MT-4, and HS-1 (30); HuT102 (43); and SP cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics; in addition, the 1657, 3614, A212, SP, FS, and EG cultures were supplemented with interleukin-2.

Genomic DNA was extracted from the HTLV-transformed cell lines by using the QIAamp DNA mini or blood kit (QIAGEN). Genomic DNAs were isolated from PBLs of Japanese ATL patients; the isolates are designated Ptnt1, Ptnt2, Ptnt4, Ptnt5, PtntAK003, PtntAK004, PtntAK005, and PtntAK006.

Amplification and sequencing of the *pol* gene. The *pol* gene was PCR amplified in two overlapping segments by using HotStarTaq DNA polymerase (QIAGEN). Nucleotides (nt) 2473 to 3856 (1,384 bp) comprised the 5' segment, and nt 3243 to 4341 (1,099 bp) comprised the 3' segment. At least two independent PCR amplifications of the target sequence were performed. PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen), and a minimum of two insert-containing TOPO vectors from each PCR amplification were sequenced. In some instances, direct sequencing of the patient sample PCR product was also performed. The HTLV-1 virus expression vector, pCS-HTLV, was directly sequenced as a reference for all constructs currently used in our lab. The pACH infectious molecular clone was also sequenced for comparison to the published *pol* gene sequence of the CH isolate (40, 46). The region between nt 2493 and 4319 of the provirus (1,827 bp) was sequenced in all isolates. In addition, the region between nt 4319 and 5127 was sequenced in the MT-2 and C10/MJ isolates.

Phylogenetic analysis. Nucleotide sequences were aligned with the CLUSTAL W program. Phylogenetic trees were constructed by the neighbor-joining method and rooted with the *pol* sequence of the HTLV-1c Mel5 isolate as an outlier. The reliability of each branch on the neighbor-joining tree was estimated by bootstrap analysis of 1,000 samplings of the original sequence alignments. Pairwise genetic distances were estimated on each sampling by the Kimura two-parameter method.

Plasmids. The cloning and construction of the pCS-HTLV plasmid vector, which contains a full-length provirus from the CS-1 cell line, has been described previously (10). The CS-1 cell line was obtained by the cocultivation of cord blood leukocytes with irradiated HTLV-1-infected HS-1 cells. The PstI-to-SstII fragment of pCS-HTLV, containing the pX region, was replaced with the homologous fragment from MT-2 to produce the pX1MT infectious clone (11). The NotI-to-SphI fragment of pX1MT was replaced with the homologous region of pCMVHT-1Δ*env* (MT-2 RT), described below, to create the pX1MT-M infectious molecular clone. The initial pCMVHT-1Δ*env* packaging plasmid was derived from the pCS-HTLV infectious molecular clone by replacing the 5' long terminal repeat promoter with a cytomegalovirus (CMV) promoter linked to a fragment (positions 439 to 567) of the R region (9). The infectious molecular clone pACH was provided by Lee Ratner (26).

To analyze the phenotypic effects of HTLV-1 isolate RT variations on viral genomic replication and infectivity, we replaced portions of the pCMVHT-1Δ*env pol* gene with the homologous region of selected isolates. The 2,366-bp BglII-to-SphI sequence (nt 2762 to 5127) of pACH was ligated into pCMVHT-1Δ*env* to create pCMVHT-1Δ*env* (CH RT). The 1,876-bp KpnI-to-SphI region (nt 3252 to 5127) of pCMVHT-1Δ*env* was replaced with the homologous regions of C10/MJ and MT-2 to create pCMVHT-1Δ*env* (C10/MJ RT) and pCMVHT-1Δ*env* (MT-2 RT), respectively. It is worthwhile to note that the RT polypeptide sequence encoded by pCMVHT-1Δ*env* (C10/MJ RT) is identical to the MT-2 RT sequence (see Fig. 2). The KpnI-to-XbaI region (nt 3252 to 4081) of pCMVHT-1Δ*env* (C10/MJ RT) was replaced with the homologous fragments from PCR-amplified EG, Ptnt1, and Ptnt4 DNAs to construct the pCMVHT-1Δ*env* (EG RT), pCMVHT-1Δ*env* (Ptnt1 RT), and pCMVHT-1Δ*env* (Ptnt4 RT) plasmid vectors. The 1,046-bp XbaI-to-SphI fragment (nt 4081 to 5127) of pCMVHT-1Δ*env* was replaced with the homologous region of MT-2 to create pCMVHT-1Δ*env* (3614 RT). Site-directed mutagenesis was used to construct pCMVHT-1Δ*env* RT Q463R. The sequences of all plasmid constructs were confirmed by sequencing.

Single-cycle replication and spreading-infection assays. Infectivity was measured by means of a single-cycle replication assay that has been described previously (9). In brief, vesicular stomatitis virus G protein (VSV-G)-pseudotyped virus-like particles were generated by cotransfection of 293T cells with a pCMVHT-1Δ*env*-derived packaging vector, a VSV-G Env expression vector (pCMV-VSV-G), and a reporter vector (pHTC-GFPluc). The reporter vector expresses a surrogate HTLV-1 genomic mRNA containing an internal CMV promoter and luciferase reporter gene that is packaged into VSV-G-pseudotyped virions. Virus-containing supernatants were collected 40 h after transfection and cleared by low-speed centrifugation and filtration through 0.45-μm-pore-size low-protein-binding filters (Millipore). Gag protein in the virus-containing supernatants was quantified using an HTLV-1 p19 (MA) enzyme-linked immunosorbent assay (ELISA) kit (Zeptometrix). The surrogate HTLV genome, containing the luciferase reporter gene, was transduced into HeLa cells by infection with 1.0 ml of the virus-containing supernatant supplemented with 5 μg/ml Polybrene. Three days after infection, HeLa cells were lysed in GLO lysis buffer (Promega). An equal volume of the cleared cell lysate was mixed with Bright GLO luciferase assay reagent (Promega), and luciferase activity was measured from triplicate readings on a luminometer. Viral infectivity was measured as the relative light units per picogram of p19 antigen present in the virus supernatant used for infection.

Spreading-infection assays were initiated by transfection of FRhL-B5 cells by calcium phosphate coprecipitation and glycerol shock (15%, vol/vol). Cells were diluted 1:5 at 3- to 4-day intervals after transfection until the cytopathic effects of virus infection were observed (12). To measure virus production, cell supernatants were collected at each passage for HTLV-1 p19 ELISA.

Nucleotide sequence accession numbers. The accession numbers of the isolates used in this study are as follows: 1657, EF076680, AMB66539, and AMB66540; 3614, EF076681, AMB66541, and AMB66542; 3669, EF076682, AMB66543, and AMB66544; A212, EF076683, AMB66545, and AMB66546; EG, EF076688, AMB66555, and AMB66556; FS, EF076689, ABM66557, and ABM66558; SP, EF076702, ABM66583, and ABM66584; HuT102, EF076691, ABM66561, and ABM66562; C8166, EF076686, AMB66551, and AMB66552; MT-2, EF076692, ABM66563, and ABM66564; MT-4, EF076693, ABM66565, and ABM66566; C10/MJ, EF076684, AMB66547, and AMB66548; C91/PL, EF076685, AMB66549, and AMB66550; CS-1, EF076687, AMB66553, and AMB66554; HS-1, EF076690, ABM66559, and ABM66560; Ptnt1, EF076694, ABM66567, and ABM66568; Ptnt2, EF076695, ABM66569, and ABM66570; Ptnt4, EF076696, ABM66571, and ABM66572; Ptnt5, EF076697, ABM66573, and ABM66574; PtntAK003, EF076698, ABM66575, and ABM66576; PtntAK004, EF076699, ABM66577, and ABM66578; PtntAK005, EF076700, ABM66579, and ABM66580; PtntAK006, EF076701, ABM66581, and ABM66582; RKI3-Ger, AF042071; SI-1 B, AF139170; WHP, AF259264; HS-35, D13784; TSP-1, M86840; ATK-1, J02029; RD-1, L10341; Boi, L36905; EL, S74562; YS, U19949; BRRP, AY563953; BRRP438, AY563954; and Mel5, L05234. The sequence of HTLV-1_{CH} is not present in GenBank and was transcribed from reference 46.

RESULTS

Nucleotide sequence analysis. Proviral *pol* gene sequences were amplified and sequenced from genomic DNAs isolated from 14 HTLV-1-transformed cell lines and from PBLs of eight ATL patients. The *pol* regions of the infectious molecular clones pCS-HTLV and pACH were also sequenced. HTLV-1 *pol* gene sequences available in GenBank were included in the sequence analysis; these previously published isolates were from primary sources (Boi, RKI3-Ger, YS, ATK-1, BRRP, BRRP438, WHP, and Mel5) (4, 6, 14, 50, 51) and cell cultures (SI-1 B, HS-35, CH, RD-1, EL, Mel5, and TSP-1) (1, 19, 22, 24, 39, 50, 59). The WHP, BRRP, and BRRP438 sequences are

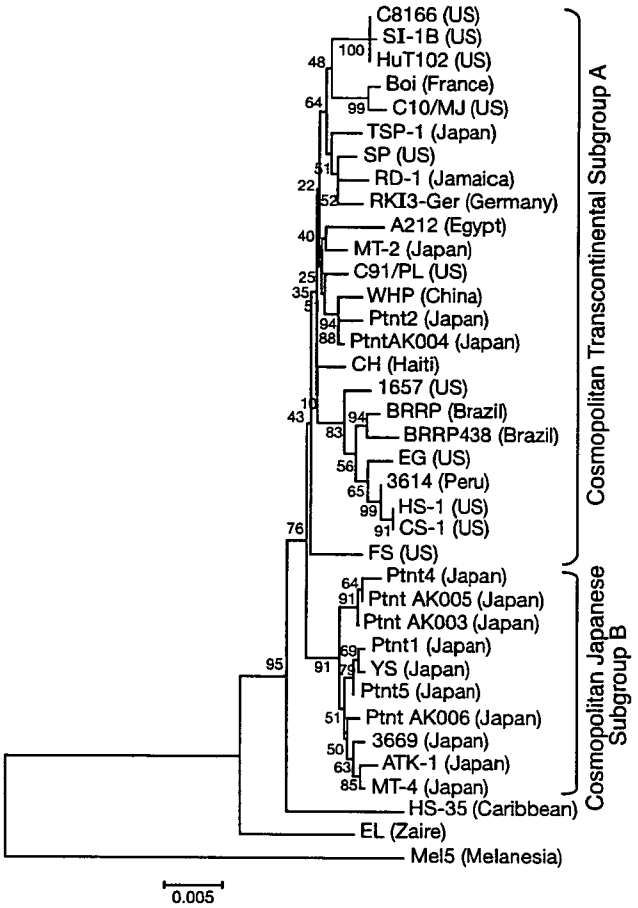

FIG. 1. Phylogenetic tree of HTLV-1 *pol*. The phylogenetic tree was constructed by the neighbor-joining method, based on the 1,827-bp RT-encoding regions of the *pol* gene. The tree was rooted by assuming the *pol* gene sequence of the HTLV-1c Mel5 isolate as the outgroup. The bootstrap test of phylogeny was used to determine tree branch reliability; values are indicated at each node.

presumed to be from uncultured isolates, but this information was not specified in their GenBank entries. In addition, uncultured PBMC, cultured PBMC, and a transformed T-cell line are all listed as sources of Mel5 genomic DNA, but it is not clear which source provided the template for *pol* sequencing. The published Mel5 *pol* gene includes two missense mutations as well as a premature stop codon in the *pol* reading frame, which would clearly result in the synthesis of a defective RT. These mutations could represent sequencing errors or could reflect somatic or reverse transcription errors.

The *pol* gene sequence of pCS-HTLV was identical to the homologous provirus sequence in CS-1 and HS-1 cells (Fig. 1). The CS-1 cell line was derived by coculture of cord blood lymphocytes with HS-1 cells (28). Likewise, the pACH *pol* sequence was identical to the previously published sequence of the pCH molecular clone (46) from which it was derived (26), indicating that no mutations of this sequence occurred during construction of the clones. No genetic differences were observed when we compared the *pol* sequence of HuT102 with that of C8166, both of which originated from the same patient. The HuT102 T-lymphoblast cell line was established in 1977 from the tumor cells of an ATL patient (16, 42). Two years later, PBLs from this patient were used to establish the HTLV-transformed cell line, CTCL-3 (42, 43), and a clone of cells, CR-M2, isolated from CTCL-3 cells was cocultivated with cord blood leukocytes to establish C8166 (also called C63/CR_{II}-2 or C81-66-45) (48). Together, these results agree with other observations of low sequence diversity between HTLV-1 isolates separated in time by in vivo passage (17).

Phylogenetic analyses have been useful for studying worldwide dissemination of the virus and categorizing HTLV-1 isolates into the three major lineages: Cosmopolitan (HTLV-1a), Central African (HTLV-1b), and Melanesian (HTLV-1c) (3, 5, 18, 19, 29, 34, 35, 49, 57). In addition, the Cosmopolitan group has been further subdivided into five currently accepted subgroups: Transcontinental (A), Japanese (B), West African/Caribbean (C), North African (D), and Black Peruvian (E) (38, 54, 58). Although the level of nucleotide sequence diversity among the HTLV-1 *pol* genes used in our analysis was typically low, it was sufficient to construct a phylogenetic tree capable of resolving subgroups within the Cosmopolitan lineage. Our phylogenetic analysis of *pol* nucleotide sequences from the aforementioned isolates showed that they all belonged to the Cosmopolitan lineage (HTLV-1a). Within this lineage, the transformed cell line isolates 1657, 3614, A212, C10/MJ, C91/PL, EG, FS, HS-1/CS-1, and HuT102/C8166 and patient sample isolates Ptnt2 and PtntAK004 belonged to the Transcontinental subgroup (HTLV-1aA), while the HTLV-1 provirus of the 3669 cell line and patient isolates Ptnt1, Ptnt4, Ptnt5, PtntAK003, PtntAK005, and PtntAK006 belonged to the Japanese subgroup (HTLV-1aB).

All of the Cosmopolitan nucleotide sequences analyzed (including those already available in GenBank) were very similar to one another in the RT-coding region, differing from one another by less than 2.5%. There were 140 sites of variation among the Cosmopolitan isolates, with 55 positions variable in more than one isolate; i.e., 85 variations were unique to one HTLV-1a isolate. Nucleotide variations favored transitions between guanine (G) and adenine (A) (39%) about as much as between cytosine (C) and thymidine (T) (39%). These are consistent with the previously noted error bias of HTLV-1 RT (6, 8, 27). The sequence context of these substitutions does not immediately suggest the activity of any known host restriction factor, such as APOBEC3G. Most of the observed genetic changes were synonymous substitutions, which suggests that positive selection of the Pol amino acid sequence occurs.

Deduced RT amino acid sequences. The consensus HTLV-1a RT amino acid sequence was generated from 23 Transcontinental isolates, 10 Japanese isolates, and 1 West African/Caribbean isolate, which gave it a Transcontinental character. No amino acid variations specific to either cell culture or primary HTLV-1 isolates were observed. As noted in previous studies that examined the *env* or LTR sequences, there was no correlation between isolate genotype and specific HTLV-associated pathologies (27). Instead, sequence similarity is generally seen between isolates of a common geographical origin, regardless of the patient's clinical status.

The most common variation from the consensus sequence was exclusive to Japanese subgroup isolates, which could be distinguished from Transcontinental isolates by the presence

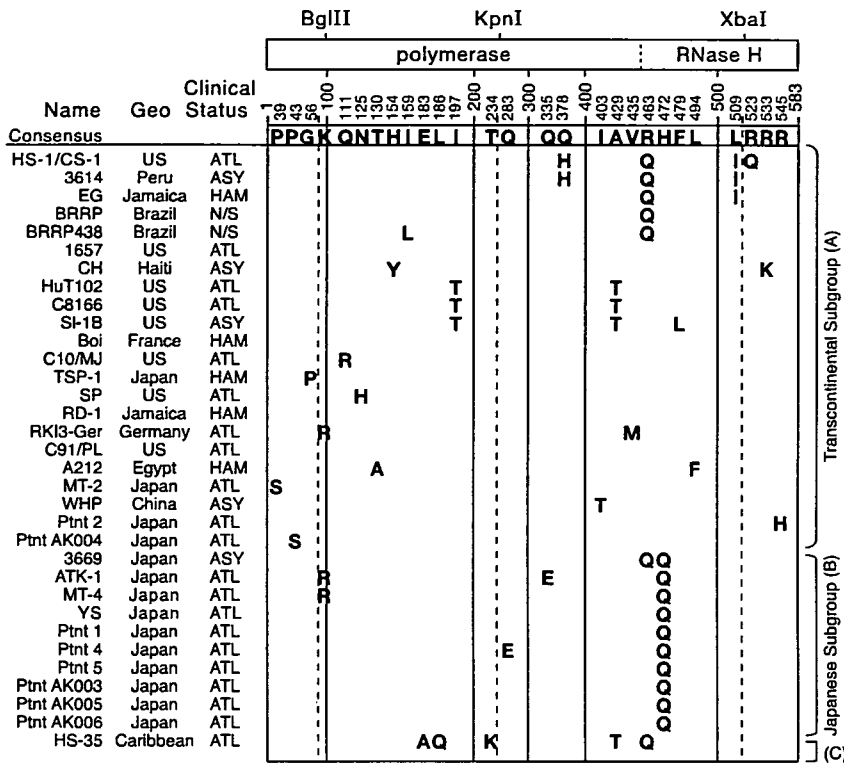

FIG. 2. Amino acid variations in the RTs of HTLV-1a isolates are mostly random. An alignment of RT amino acid sequences from various HTLV-1a isolates is shown. Each column indicates a site of amino acid variation. The HTLV-1a consensus amino acid is shown at the top of the column, and isolates with variations are presented underneath. Numbering is based on Gly166 of the *pro* open reading frame as the N-terminal residue of RT (20) and Leu583 as the approximate C-terminal residue (33). The locations of restriction endonuclease sites in the *pol* gene used for cloning and the junction between the polymerase and RNase H domain are indicated relative to the sites of variation. The sequence of the FS isolate was not included because it contains three stop codons. Isolates are organized by their Cosmopolitan lineage subgroup: Transcontinental (A), Japanese (B), and West African/Caribbean (C).

of a glutamine at position 472 of RT (Fig. 2). Of the less frequently seen variations, a histidine residue at position 378 in place of the consensus glutamine and an isoleucine at position 509 instead of leucine were variations observed in HS-1/CS-1 (United States) and 3614 (Peru). Likewise, a threonine in place of the consensus isoleucine at amino acid residue 197 was present in U.S. isolates SI-1 B and HuT102/C8166. Several other variations were seen in more than one Cosmopolitan subgroup. A few Transcontinental and Japanese isolates, besides those sequenced here, have arginine at position 100 of RT (45, 46), though lysine is the consensus residue at this position. A glutamine at residue 463 of RT was prevalent in HTLV-1aA isolates from the Caribbean region and South America (BRRP, BRRP438, 3614, EG, and HS-35) but was also observed in Japanese subgroup isolate 3669 and in the HTLV-1c isolate Mel5 (not shown). The threonine residue variation at position 429 of RT was seen in SI-1 B and HuT102/C8166 of the Transcontinental subgroup, as well as in the HTLV-1aC subgroup isolate HS-35.

Phenotypic effects of variation on viral infectivity. To determine whether certain amino acid variations in RT correlated with virus infectivity, we used a sensitive, quantitative single-cycle infection assay to measure reporter gene transduction (9). To ensure a similar viral context in which to compare the effects of RT variations, the *pol* gene in the CS-1-derived vector pCMVHT-1Δenv (9) was replaced with *pol* gene fragments from the MT-2, C10/MJ, CH, 3614, Ptnt1, Ptnt4, and EG proviruses. These isolates were selected because they either encoded the consensus RT sequence (MT-2 and C10/MJ), encoded the RT of the other common HTLV-1 infectious molecular clone (CH), represented the consensus Japanese subgroup RT (Ptnt1) or a variant thereof (Ptnt4), or clustered with CS-1 on the phylogenetic tree (3614 and EG).

Vectors encoding an RT that closely matched the consensus RT sequence, such as MT-2, C10/MJ, and the prototypical Japanese subgroup sequence Ptnt1, displayed the highest titers in a single-cycle infection assay (Table 1). In general, variations from the consensus sequence resulted in decreased viral replication. The presence of a glutamate at amino acid 283 of the Ptnt4 RT significantly decreased viral replication in comparison to the activity of either the Ptnt1 or consensus RT. One or both of the consensus sequence variations present in the CH isolate, Tyr154 and Lys533, reduced viral infectivity nearly threefold relative to the consensus RT. The variations seen in a cluster of U.S., Caribbean, and South American isolates had a significant effect on viral replication. The relative infectivity of these isolates decreased from 36% (EG) to almost 14% (CS-1) of that of the MT-2 RT isolate activity as a result of variations at a combination of positions in the connection and RNase H domains. A derivative of CS-1, where the glutamine

TABLE 1. Single-cycle infection assay of HTLV-1 vectors encoding various RTs

Isolate[a]	Amino acid variation from consensus RT sequence:								Relative infectivity (%)[b]
	H-154	Q-283	Q-378	R-463	H-472	L-509	R-523	R-533	
MT-2									100
C10/MJ									84
Ptnt1					Q				135
Ptnt4		E			Q				37
CH	Y							K	42
EG				Q		I			36
3614			H	Q		I			37
CS-1 (Q463R)			H			I	Q		24
CS-1			H	Q		I	Q		13

[a] The RT-encoding *pol* region of the indicated HTLV-1 isolates and the CS-1 Q463R mutant were cloned into pCMVHT-1Δ*env*.
[b] Normalized to MT-2. The mean of results from duplicate infections was determined for each isolate. CS-1 and MT-2 were tested 10 times. Most other isolates were tested a minimum of three times.

at position 463 was mutated to the consensus arginine (Q463R), had a twofold-higher relative infectivity than the CS-1 clone, in agreement with our observation that RT sequences closer to the consensus sequence generally display a higher infectivity.

The difference in replication between virus with the consensus sequence RT, represented by MT-2, and the CS-1 variant was further confirmed in a spreading-infection assay. FRhL-B5 cells were transfected with pX1MT or pX1MT-M, encoding the CS-1 and MT-2 RT sequences, respectively, and virus production was monitored by p19 ELISA. As seen in Fig. 3, virus production in cell cultures transfected with pX1MT typically peaked at around 3 weeks after transfection, while peak virus production in cultures transfected with pX1MT-M consistently occurred after only 2 weeks.

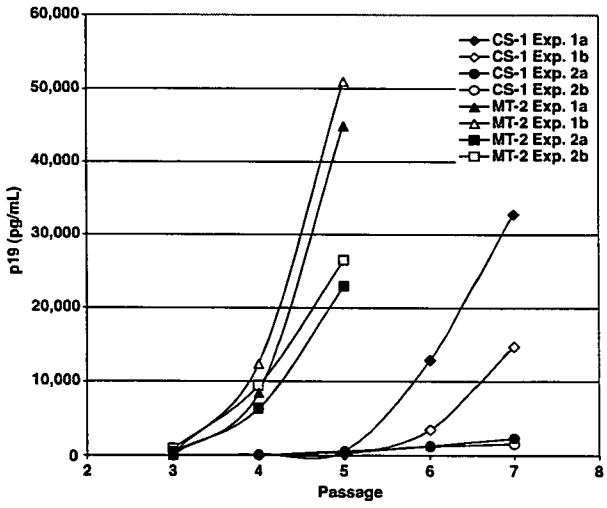

FIG. 3. Replication kinetics in spreading-infection assays correlate with RT sequence. A comparison of the virus infection kinetics between the HTLV-1 infectious molecular clones encoding the CS-1 and MT-2 isolate RT sequences is shown. For each experiment, infections were initiated in duplicate by transfection of FRhL-B5 cells with cloned provirus. Cells were passaged at a 1:5 dilution every 3 to 4 days. At each passage, virus expression was monitored by HTLV-1 p19 ELISA of the culture supernatant. The clone containing MT-2 RT consistently achieved peak virus production two passages before the CS-1 RT clone.

DISCUSSION

To determine if the low infectivity of HTLV-1 virions in vitro is strictly a feature of viruses produced by HTLV-transformed cell lines and the infectious molecular clones derived from these cell lines, we performed a genotypic and phenotypic comparison of the HTLV-1 *pol* genes from primary isolates, transformed cells, and molecular clones. A phylogenetic analysis of these isolates showed that they belonged to the Cosmopolitan lineage and varied from other HTLV-1a isolates by less than 2.5%. A few of these nucleotide sequence variations also translated into changes in the amino acid sequence of RT, suggesting positive selection for RT function. Some variations from the consensus sequence were shared among several isolates, most notably the glutamine at position 472 in all Japanese subgroup isolates, but most of the variations were unique to a single HTLV-1 isolate. Amino acid variations did not correlate with virus isolates from transformed cell lines versus primary sources, nor did variations correlate with disease status. Finally, no mutations in the *pol* gene sequence occurred during construction of the molecular clones. Therefore, the *pol* gene sequences of HTLV-transformed cell lines and infectious molecular clones appear to accurately represent primary HTLV-1 isolates.

The relationship between Pol amino acid sequence and HTLV-1 infectious titer was examined by inserting *pol* gene sequences from different virus isolates into the packaging plasmid pCMVHT-1Δ*env* for single-cycle infection assays or into the infectious molecular clone pX1MT for spreading infections. Recombinant viruses whose *pol* genes most closely matched the consensus sequences, such as MT-2, C10/MJ, and Ptnt1, gave the highest titers. Viral titers decreased roughly in proportion to the number of amino acid variations from the consensus sequence that were present. These data suggest that it is unlikely that a very-high-titer variant of HTLV-1 with a unique *pol* gene exists, analogous to the high-titer beta strain of RSV (55). The differences in titers for HTLV-1 clones with variant *pol* genes is reminiscent of the difference in viral titers observed for highly pathogenic SIVmne027 and slow-replicating SIVmneCl8, which differ functionally as the result of a single K412E mutation in the connection domain of RT (25, 41; Jason Kimata, personal communication).

There have been only two reports that made an attempt to

quantify infectious titers for cell-free HTLV-1 particles. In the first, Fan et al. used virions produced from MT-2 cells, and measured infection by PCR of nascent reverse transcription products (minus-strand strong-stop DNA) (15). Those authors calculated that one in 10^6 particles were infectious, a value that is at least 1,000-fold lower than the specific infectivity of HIV-1 particles. However, the specific infectivity for HTLV-1 that is based on MT-2 virions is likely to underestimate the actual value by about 100-fold, because MT-2 cells contain seven defective proviruses and one full-length provirus. Thus, assuming that all proviruses are expressed and packaged, which appears to be likely from RNA analyses (21, 53), only one in 64 particles would contain a dimer of full-length genomic RNA. This would put the actual specific infectivity of HTLV-1 particles at closer to one in 10^4. In a later study, we used VSV-G-pseudotyped viral vectors to show that HTLV-1 particles gave about 1,000-fold-lower titers than HIV-1 particles (9). These calculations were based on HTLV-1 vectors that contain the CS-1 RT sequence, which give about 10-fold-lower titers than the vectors containing a consensus RT. Calculations based on results from the latter HTLV-1 vectors indicate that HTLV-1 particles are only 100-fold less infectious than HIV-1 particles in cell-free infection experiments. These data indicate that the lower relative titers for pseudotyped HTLV-1 particles are manifested at a postentry step and are consistent with the results of Fan et al. (15), which suggested that this reflects a difference in reverse transcription. The data presented here suggest that cell-free infection in vitro with HTLV-1 is not as low as previously thought. The new HTLV-1 vectors, which encode a consensus RT, increase the sensitivity of in vitro infection assays by 10 times, and this should significantly enhance future studies with HTLV-1 particles containing HTLV-1 Env.

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Darunavir, a conceptually new HIV-1 protease inhibitor for the treatment of drug-resistant HIV

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Abstract—Our structure-based design strategies which specifically target the HIV-1 protease backbone, resulted in a number of exceedingly potent nonpeptidyl inhibitors. One of these inhibitors, darunavir (TMC114), contains a privileged, structure-based designed high-affinity P2 ligand, 3(*R*),3a(*S*),6a(*R*)-bis-tetrahydrofuranylurethane (bis-THF). Darunavir has recently been approved for the treatment of HIV/AIDS patients harboring multidrug-resistant HIV-1 variants that do not respond to previously existing HAART regimens.

The AIDS (acquired immunodeficiency syndrome) epidemic has become one of the most pressing medical concerns of our time.[1] The World Health Organization (WHO), as of 2006, estimated that over 40 million people are infected with HIV (human immunodeficiency virus), the causative agent of AIDS.[2] During replication in the HIV life-cycle, *gag* and *gag-pol* gene products are produced as precursor polyproteins which are subsequently processed by a virally encoded protease to provide structural proteins (p17, p24, p9 and p7) and essential viral enzymes, including protease (PR), reverse transcriptase (RT) and integrase (IN).[3] All three retroviral enzymes have been identified as potential drug targets. Specifically, the critical function of HIV protease has made it an important target for the treatment of HIV/AIDS. The approval of the first protease inhibitor (PI), saquinavir and its introduction into highly active antiretroviral therapy (HAART), with reverse transcriptase inhibitors, led to significantly enhanced HIV management and improved the quality of life of HIV/AIDS patients.[4]

Since the advent of saquinavir, a number of PIs have been introduced in the regimens of HAART. Thus improved HAART regimens have shown reduced viral load, increased CD4+ T-cell counts[5] and drastically lowered AIDS-related deaths in the US and industrialized nations.[6] While HAART proved to be a large step forward, there are still serious drawbacks with the first generation anti-protease therapeutics. These include: (1) severe side effects and drug toxicities, (2) higher therapeutic doses due to 'peptide-like' character, (3) costly synthesis which leads to high treatment cost, and perhaps the most alarming, (4) the rapid emergence of drug resistance. Indeed, the emergence of multidrug-resistant HIV strains has greatly compromised current HAART regimens. It has been reported that treatment failure has ultimately occurred in at least 40–50% of patients, who initially achieved favorable viral suppression with HAART to undetectable levels.[7] Furthermore, persistent viral replication (plasma HIV RNA > 500 copies/mL) has been reported under HAART in 10–40% of antiviral therapy-naive individuals as a result of transmission of drug-resistant HIV-1 variants.[8] The management and effective treatment options for HIV/AIDS clearly depend upon the development of PIs and other novel anti-HIV therapeutics, which can effectively combat drug-resistant HIV strains, possess better pharmacokinetic properties, have no or less toxicities, and come at a reduced cost of synthesis.

Keywords: Protease inhibitors; Darunavir; Design and synthesis.
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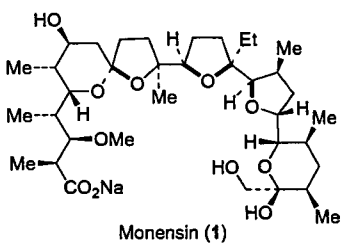
1. Design of nonpeptide ligands to eliminate peptidic character

Our initial investigation primarily focused on reducing peptidic features, molecular weight, and structural complexity of protease inhibitors. In this context, we have designed a number of nonpeptidic high-affinity ligands for the HIV protease substrate binding site based upon various available three-dimensional structures of the protein-ligand complex.[9] Particularly, we planned to design conformationally constrained nonpeptidic molecules of a cyclic or heterocyclic nature to maximize the active site interactions. One of the important elements in our design strategy is the incorporation of a stereochemically defined and conformationally constrained cyclic ether template that could replace peptide bonds and mimic their biological mode of action by retaining critical interactions in the active site.[9,10] The idea of designing cyclic ether-based ligands emerged from our observation that numerous bioactive natural products are comprised of these cyclic ether motifs. Of particular interest, ionophore antibiotic, monensin (**1**, Fig. 1)[11] and platelet activating factor antagonist, ginkgolide B (**2**),[12] which feature these cyclic ether subunits, do not suffer from oral bioavailability problems inherent to peptide and peptidomimetic-based inhibitor drugs.

Indeed, our structure-based design strategy led to the development of a number of cyclic ether-derived nonpeptide P2-ligands for the HIV protease substrate binding site. We have documented an intriguing potency enhancing effect of 3(*S*)-tetrahydrofuranyl urethane in inhibitors containing a hydroxyethylene isostere or a hydroxyethylsulfonamide isostere.[13] Incorporation of 3(*S*)-tetrahydrofuran into a (*R*)-(hydroxyethyl)sulfonamide isostere afforded a highly potent inhibitor which later became amprenavir.[14] It is noteworthy to mention that the tetrahydrofuranyl subunit is inherent in both monensin and various ginkgolides (Fig. 1).



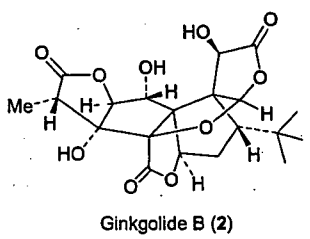


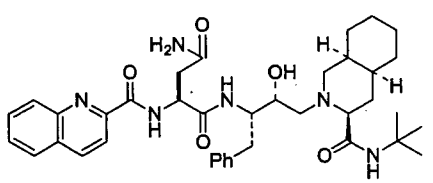
Figure 1. Structures of monensin and ginkgolide B.

2. Design of bis-THF: an inspiration from bioactive natural products

Analysis of a number of protein-ligand X-ray structures of 3(*S*)-tetrahydrofuranyl urethane-bearing inhibitors revealed weak hydrogen bonding between the tetrahydrofuranyl oxygen and the main chain aspartic acids (Asp-29 and Asp-30) as well as van der Waals interactions in the S2-site. Based upon this structure, our subsequent objective became to design a ligand that would maximize the hydrophobic and hydrogen bonding interactions with the residues in the S2-site. Our critical analysis of the saquinavir (**3**)-bound protease X-ray crystal structure led us to design and develop a stereochemically defined bicyclic tetrahydrofuran (bis-THF) ligand that appeared to effectively hydrogen bond with both Asp-29 and Asp-30 NHs. The bicyclic ring of the bis-THF is also poised to offset the loss of P3 hydrophobic binding of the quinoline ring in saquinavir. Inhibitor **4** (enzyme IC_{50} of 1.8 nM and antiviral CIC_{95} of 46 nM, Fig. 2) has shown improved aqueous solubility, reduced peptidic features and molecular weight compared to saquinavir.[9] Subsequent detailed studies established that the stereochemistry, the position of the oxygen atoms, ring sizes and substituents are all essential for potency.[9] The X-ray crystal structure of **4**-bound protease revealed that the bis-THF ring oxygens are involved in effective hydrogen bonding interactions with both the backbone NH's of Asp-29 and Asp-30 present in the S2 subsite. In essence, the bis-THF ligand, a subunit of ginkgolides (bicyclic acetal) remarkably mimics the binding of the P2 asparagine carboxamide and the P3 quinaldic amide carbonyl of saquinavir.

3. Design and development of 'darunavir' to combat drug resistance

Our analysis of protein-ligand complexes of wild-type and mutant proteases and an overlay of the corresponding protein backbones showed only minimal distortion of the backbone conformation, particularly in the active site of the protease.[15] This is also apparent in other reported high resolution X-ray structures of related inhibitor/ligand complexes.[16] This observation led us



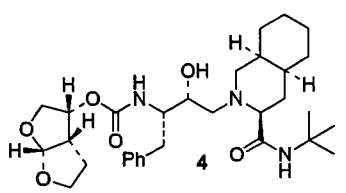


Figure 2. Structures of saquinavir and a bis-THF inhibitor.

to speculate that an inhibitor making extensive hydrogen bonding interactions with the protein backbone of the wild-type enzyme will also maintain potency against mutant strains. Our inhibitor design strategy to combat drug-resistance then focused on optimizing of the ligand-binding site interactions so as to make maximum interactions in the active site, including hydrophobic, electrostatic and most critical, hydrogen bonding with the backbone atoms located in the S2 to S2′-subsites of protease. As mentioned previously, inhibitor **4**-bound X-ray structure of HIV protease revealed that while the P2 bis-THF ligand makes extensive interactions including backbone hydrogen bonding in the S2-subsite, similar hydrogen bonding in the S2′-site are mostly absent.

With the objective of designing an inhibitor that can make robust hydrogen bonding throughout the S2-S2′-subsites, we investigated the effect of a P2 bis-THF ligand with a number of different isosteres.[9] Incorporation of the P2 bis-THF in (*R*)-(hydroxyethyl) sulfonamide isosteres led to a number of potent PIs, with impressive drug resistance profiles. For instance, inhibitor **5** (UIC-PI or UIC94003 and later TMC-126), incorporating a P2 bis-THF and a *p*-methoxysulfonamide as the P2′ ligand exhibited remarkably potent enzyme inhibitory potency (K_i value 14 pM, Fig. 3) and antiviral activity (ID_{50} value of 1.2 nM) in CEM cell lines.[17] It was also profiled against numerous mutant HIV proteases. The K_i was less than 100 pM in every case and the K_{imut}/K_{iwt} was no greater than five. This indicates a low level of resistance even for enzymes with multiple mutations which have been shown to be resistant to clinically active inhibitors. TMC-126 has shown a remarkable drug resistance profile and has maintained high potency in the presence of human serum albumin.[17]

Inhibitor **6** (UIC 94017, later known as TMC-114) with a bis-THF as the P2 ligand and *p*-aminosulfonamide as the P2′ ligand has also exhibited very impressive inhibitory properties. It displayed an enzyme inhibitory potency (K_i) of 16 pM and an antiviral ID_{50} of 4.7 nM in CEM cell lines. It showed an antiviral ID_{90} of 10 nM, and a $TD_{50} > 100$ μM in cell culture assays.[18] Inhibitor-bound X-ray structure analysis revealed that both P2 and P2′-ligands of inhibitor **6** are involved in extensive hydrogen bonding with the protein backbone. This may be responsible for its potency and wide-spectrum activity against multi-PI-resistant HIV-1 variants. It was tested against a panel of 20 HIV variants resistant to current PI's, but there was no greater than a 5-fold increase in ID_{50} values, indicating it remained active against the resistant strains. In addition, the P2′-amine group provided more favorable pharmacokinetic properties compared to the P2′-methoxy group in inhibitor **5**. Subsequently, it was selected for clinical development and renamed darunavir.[19]

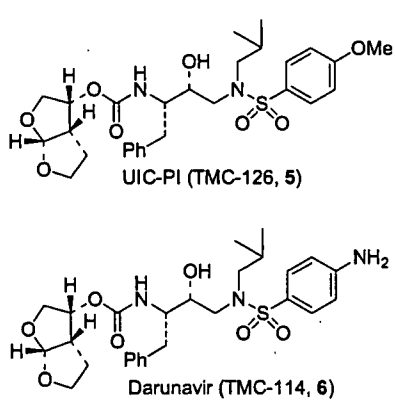


Figure 3. Structure of darunavir and inhibitor **5**.

Clinical development of darunavir was conducted by Tibotec-Virco, Belgium.[18b] POWER 1 and POWER 2 clinical trials of ritonavir-boosted darunavir (DRV/r) were carried out with treatment-experienced patients who were no longer benefiting from available PIs. Over a period of six months, both studies showed that combination therapy using DRV/r led to a reduction in viral load below 50 copies/mL in 45% of participants compared with only 12% of participants given another available PI. CD4+ cell counts in the DRV/r group rose by an average of 92 cells/mm^3 over the six month period compared with an average increase of 17 CD4+ cells/mm^3 for participants receiving another PI during this time.[20] POWER 3, a non-randomized, open-label trial was conducted to assess the long-term efficacy and safety of DRV/r 600/100 mg BID in treatment-experienced patients. The primary efficacy endpoint was the proportion of patients with ≥ 1 log10 reduction in HIV RNA by week 24. Reduction of HIV RNA with an efficacy endpoint of ≥ 1 log10 was observed in 65% of patients. Reductions in HIV RNA levels to < 400 copies/mL and < 50 copies/mL were observed in 57% and 40% of patients, respectively. DRV received accelerated approval by the FDA on June 23, 2006.[20] Recent studies have shown that darunavir, when used in combination with the fusion inhibitor FUZEON, can substantially increase the chances of reaching undetectable viral load.[21]

4. Recent PIs based on the bis-THF ligand

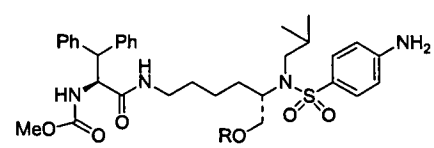
The success of Darunavir and the evidence indicating the importance of the bis-THF P2 ligand has led to an expansion of the 'backbone binding concept', and produced several novel and active PIs. Ritonavir has recently been modified with the addition of the P2-bis-THF ligand, and initial SAR results revealed a new potent inhibitor.[22] Introduction of a fused benzodioxolane and other related functionalities as P2′ sulfonamides have shown significant potency enhancement and drug-resistance properties.[23] GlaxoSmithKline researchers have investigated various structural modifications at the P1 and P1′ positions of inhibitors containing a bis-THF group as the P2-ligand and a benzodioxolane sulfonamide as the P_2'-ligand.[24] One of the inhibitors has shown IC_{50} values in the single digit nanamolar range as well as K_i's in the femtomolar range.[25] This inhibitor was later renamed brecanavir and had undergone extensive clinical studies. However

brecanavir development was later terminated reportedly due to its difficulty to formulate. Tibotec researchers have discovered a new series of fused benzoxazole and benzothiazole ligands to fit the S2′ domain. These new inhibitors have shown broad-spectrum antiviral activity against PI resistant mutants, as well as excellent pharmacokinetic properties.[26]

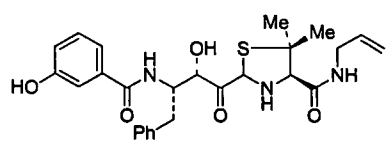
5. Beyond the bis-THF ligand and darunavir: recent developments in protease inhibitors

Recently, Procyon pharmaceuticals has reported a new class of protease inhibitors based on L-lysine. Uniquely, two novel sulfonamide based PIs (PL-100, **7** and PPL-100, **8**, Fig. 4) have displayed good protease inhibitory activity.[27] They have shown enzyme inhibitory activity less than 20 nM, but their preliminary cross-resistance results are very impressive. Against 14 viral stains from highly PI-experienced patients, PL-100 showed a 4.5 average fold-increase in IC_{50} values.[28] The phosphate ester prodrug, PPL-100 (**8**), showed a superior pharmacokinetic profile.[29] AG-001859, **9** is another recently identified compound which exhibited potency against resistant strains of HIV.[30] This new compound is an allophenylnorstatin-based PI, and has shown K_i's for wild-type and mutant proteases as low as 0.1 nM. When AG-001859 was tested against 44 PI-resistant HIV-1 isolates, it displayed excellent potency with a median EC_{50} of 34 nM (range 5.3–420 nM).[30] AG-001859 was selected for further testing and has started a phase 1 clinical trial.

We recently developed a new P2 ligand, based upon the 'backbone binding' design concepts. Inhibitor **10** contains a stereochemically defined bicyclic hexahydrocyclopentanofuran as a P2 ligand and a hydroxymethylphenylsulfonamide group as the P2′ ligand (Fig. 4) It has shown potent antiviral activity (K_i = 4.5 pM, IC_{50} = 1.8 nM) and effective drug-resistance properties against a panel of multi-PI-resistant HIV-1 isolates with IC_{50} values ranging from 4–52 nM.[31]



7 R = H (PL-100)
8 R = $OPO(OH)_2$ (PPL-100)



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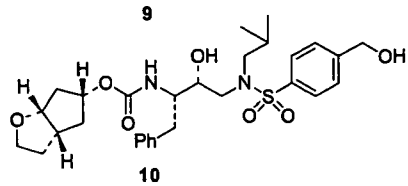

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Figure 4. Structures of recent protease inhibitors.

6. Future directions of anti-protease treatment

The future management of HIV/AIDS should rely upon the development of therapies that are less toxic and more effective in combating drug-resistance. Since protease inhibitors are very important components of current HAART regimens, design and development of new PIs with improved pharmacological properties and better drug-resistance profiles are of great importance. In this context, our design strategies target the active site protein-backbone as there is minimal change in the backbone conformations of the wild-type and mutant proteases. Of particular note, we have developed a new generation of PIs bearing a structure-based designed bis-THF ligand that effectively fills in the hydrophobic pocket and maximizes hydrogen bonding interactions with the backbone atoms of the S2-site. A number of bis-THF-derived inhibitors are exceedingly potent and have maintained very impressive potency against multidrug-resistant HIV-1 variants. One of these inhibitors, darunavir, has been recently approved by the FDA as the first treatment of drug-resistant HIV. Our detailed structural analysis of darunavir-bound wild-type and mutant proteases have documented extensive hydrogen bonding interactions with the active site backbone atoms. This design concept targeting the backbone may serve as an important guide to combat drug resistance. Further development of novel PIs with designed functionalities is currently the focus of our ongoing investigation.

Acknowledgments

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