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Analysis of the S3 and S3′ subsite specificities of feline immunodeficiency virus (FIV) protease: Development of a broad-based protease inhibitor efficacious against FIV, SIV, and HIV *in vitro* and *ex vivo*

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ABSTRACT **The S3 and S3′ subsite binding specificities of HIV and feline immunodeficiency virus proteases (FIV) proteases (PRs) have been explored by using C_2-symmetric competitive inhibitors. The inhibitors evaluated contained (*1S, 2R, 3R, 4S*)-1,4-diamino-1,4-dibenzyl-2,3-diol as P1 and P1′ units, Val as P2 and P2′ residues, and a variety of amino acids at the P3 and P3′ positions. All inhibitors showed very high potency against HIV PR *in vitro*, and their K_i values ranged between 1.1 and 2.6 nM. In contrast to the low restriction of P3 and P3′ residues observed in HIV PR, FIV PR exhibited strong preference for small hydrophobic groups at the S3 and S3′ subsites. Within this series, the most effective inhibitor against FIV PR contained Ala at P3 and P3′. Its K_i of 41 nM was 415- and 170-fold lower than those of the inhibitors without the P3 and P3′ moieties or with the Phe at these positions, respectively. In addition, these compounds were tested against mutant FIV PRs, which contain amino acid substitutions corresponding to those in native HIV PR at homologous sites, and their efficacy of inhibition progressively increased up to 5-fold. The most potent FIV PR inhibitor was selected for examination of its effectiveness in tissue culture, and it was able to block nearly 100% of virus production in an acute infection at 1 μg/ml (1.1 μM) against HIV, FIV, and simian immunodeficiency virus. Furthermore, it was not toxic to cells, and even after 2 months of culture there was no sign of resistance development by virus. The findings suggest that inhibitors with small P3 residue may be efficacious against a broad range of HIV variants as well as interspecies PRs.**

In an effort to develop therapeutic strategies to control the progress of AIDS, HIV protease (HIV PR) has become an important target enzyme for the inhibition of viral replication. Although several competitive inhibitors of this protease have been approved or are in clinical trials (1–9), many drug-resistant mutant HIV PRs have been identified (10, 11). In addition, the drug development process has been relatively slow because of the lack of animal systems to test the effectiveness of the inhibitors.

Feline immunodeficiency virus (FIV) (12, 13) causes an immunodeficiency syndrome in cats comparable to AIDS in humans. Thus, the cat offers a potential vehicle for the development of antilentiviral agents amenable to test *in vivo*. In fact, the FIV has been used as a model for preclinical evaluations of HIV reverse transcriptase (RT) inhibitors in animals (14). The active site structures of FIV and HIV PRs are superimposable and have an identical mechanism of catalysis (15, 16). Furthermore, at least six mutated residues in HIV PR that cause drug resistance are also found in the structurally aligned native residues of FIV PR (16, 17). Kinetic studies also showed that various potent HIV PR inhibitors containing the P3 to P3′ residues (6–8, 16), including the FDA-approved drug Ro 31–8959 (Fig. 1) (6), are less-efficient inhibitors of FIV PR by a factor of 100 or more (16). Although the significance of these observations is yet to be appreciated, it suggests that FIV PR may serve as a model for drug-resistant mutant HIV PRs and may contribute to the understanding of HIV resistance to protease inhibitors. Therefore, we are developing potent inhibitors of FIV PR containing residues that bind to the S3 to S3′ region with the aim of developing broad-based therapeutic agents against AIDS that may be less prone to resistance development. An additional advantage of this strategy, if effective, is to facilitate the *in vivo* testing of candidate inhibitors in an animal system.

HIV PR and FIV PR are responsible for processing both the structural proteins of *gag* and the enzymes encoded by *pol* from their respective polyproteins (18). Although the cleavage sites between the individual proteins of *gag* and *pol* in HIV and FIV are unique, the site between the MA-CA domain of the *gag-pol* polyprotein in both viruses is identical at four of six residues from P3-P3′ [for FIV (19) and for HIV (20)]. This indicates a significant role of P3 and P3′ residues in defining substrate specificity for HIV and FIV PRs. Accordingly, for development of effective inhibitors of HIV and FIV PRs, it is important to identify and to extend our understanding of substrate and inhibitor binding in the S3 and S3′ subsites of the enzymes where binding specificities are relatively unknown.

Because the active sites of both HIV and FIV PRs are C_2-symmetric, it has been predicted that the axis of symmetry of an inhibitor with a C_2-symmetric unit would coalign with the C_2-axis of the enzymes resulting in specific inhibition (4, 5). In fact, C_2-symmetric inhibitors containing diol cores have been identified as extremely potent inhibitors of HIV PR *in vitro* (5). The x-ray crystal structure of HIV PR complexed with the inhibitor A-76889, containing (1*S*, 2*R*, 3*R*, 4*S*)-1,4-diamino-1,4-dibenzyl-2,3-diol as P1 and P1′ unit and N-protected Val as P2-P3 and P2′-P3′, also displayed the high degree of structural correspondence at P1-P3, P1′-P3′ although the *R,R* diol core bound in an asymmetric mode (21). Therefore, evaluating the binding affinities of C_2-symmetric inhibitors represents a convenient strategy for systematic probing of enzyme specificities at certain sites. The kinetic parameters of the FIV PR for peptide substrates also indicated that replacing Val at P2 position by Ile led to a significant decrease in binding

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Abbreviations: FIV, feline immunodeficiency virus; SIV, simian immunodeficiency virus; RT, reverse transcriptase.

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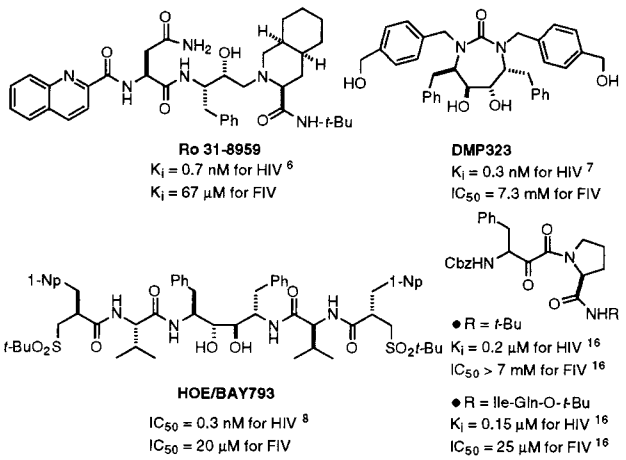


FIG. 1. Examples of HIV PR inhibitors tested as inhibitors of FIV PR.

(15). Therefore, compound **5** (Scheme 1) was chosen as a core unit. A variety of amino acids were then introduced to the core as the P3 and P3′ residues to rapidly create a number of potential inhibitors for the analysis of amino acid restriction and tolerance at the S3 and S3′ subsites of HIV and FIV PRs. Promising compounds revealed by *in vitro* analyses were then tested *ex vivo* for efficacy against FIV, SIV, and HIV infection.

MATERIALS AND METHODS

Chemical Synthesis. 1,4-Bis[(*N*-Cbz)amino]-1,4-dibenzyl-2,3-diol **1** was prepared by using a literature procedure (22). All new compounds were homogeneous by TLC and were characterized by satisfactory ^{1}H, ^{13}C NMR, and mass spectra.

Compound 2. To a suspension of diastereomeric mixture of 1,4-bis[(*N*-Cbz)amino]-1,4-dibenzyl-2,3-diol **1** (1.5 g, 2.63 mmol) in 2,2-dimethoxypropane (50 ml) was added catalytic amounts of *p*-TsOH. The reaction mixture was heated at 60°C for 5 hr and cooled to 20°C. The reaction mixture was diluted with EtOAc (200 ml), and the resulting solution was washed with sat. aq. $NaHCO_3$ and sat. aq. NaCl, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was then purified by flash chromatography (hexanes/EtOAc 80/20) to give 2,3-protected (1*S*, 2*R*, 3*R*, 4*S*)-diastereomer **2** (1.28 g, 80%) as a white solid.

Compound 4 (General Procedure for the Coupling Reaction). Compound **2** (1.20 g, 1.97 mmol) in EtOAc (75 ml) containing 10% Pd/C (400 mg) was stirred under H_2 (1atm) at 20°C for 20 hr. The reaction mixture was filtered through Celite and then concentrated *in vacuo* to give diamine **3** (664 mg, 99%) as a colorless viscous oil, which was used for coupling reaction without purification.

HBTU (1.45 g, 3.82 mmol) and then Et_3N (425 mg, 4.2 mmol) were added to a solution of diamine **3** (650 mg, 1.91 mmol) and *N*-Cbz-L-valine (961 mg, 3.82 mmol) in CH_3CN (16 ml). The reaction mixture was stirred for 15 min at 20°C under Ar and then quenched by addition of brine (60 ml) and extracted with EtOAc (4 × 50 ml). The organic layer was washed sequentially with 1 M HCl (10 ml), sat. aq. $NaHCO_3$ (10 ml), and sat. aq. NaCl (10 ml), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by flash chromatography to give compound **4** (1.16 g, 75%) as a white solid.

Compound 10 (General Procedure for Deprotection). Catalytic amounts of *p*-TsOH were added to a solution of compound **4** (55 mg, 0.068 mmol) in MeOH (3 ml). The reaction mixture was heated at 60°C for 24 hr then diluted with EtOAc (20 ml). The organic solution was washed with sat. aq. $NaHCO_3$ (5 ml) and sat. aq. NaCl (5 ml), dried over $MgSO_4$, filtered and concentrated *in vacuo* to give free diol **10** (36 mg, 69%) as a white solid.

The preparations of compound **11**-**14** were carried out by using the general procedures for coupling and deprotection.

Compound 11. In a same manner, compound **4** (1.10 g, 1.36 mmol) was hydrogenated to give compound **5** (665 mg, 99%) as a colorless viscous oil.

Compound **5** (20 mg, 0.037 mmol) was coupled to give compound **6** (27 mg, 79%) as a white solid.

Compound **6** (22 mg, 0.024) was deprotected to yield compound **11** (13 mg, 62%) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6, 80°C) δ 0.74 (3H, d, J = 4.9), 0.76 (3H, d, J = 4.9), 1.90 (1H, se, J = 6.4), 2.72–2.80 (2H, m), 3.37 (1H, s), 3.68–3.73 (3H, m), 4.10 (1H, dd, J = 8.3, 6.7), 4.33 (1H, s), 4.36–4.42 (1H, m), 5.09 (2H, s), 7.02–7.09 (1H, br), 7.10–7.41 (12H, m); ^{13}C NMR. (100 MHz, DMSO-d_6, 80°C) δ 17.1, 19.0, 30.1, 39.0, 42.5, 50.7, 58.0, 65.7, 72.5, 125.5, 125.7, 128.2, 128.5, 128.8, 129.4, 136.6, 138.8, 155.7, 168.5, 170.2; HRMS (FAB+), calculated for MCs^+ $C_{48}H_{60}N_6O_{10}Cs$ m/z 1013.3425, found m/z 1013.3447.

Compound 12. Compound **5** (68 mg, 0.13 mmol) was converted to compound **7** (80 mg, 67%) as a white solid. Compound **7** (55 mg, 0.058) was deprotected to give compound **12** (42 mg, 80%) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6, 80°C) δ 0.70 (3H, d, J = 2.4), 0.72 (3H, d, J = 2.4), 1.21 (3H, d, J = 7.0), 1.87 (1H, se, J = 6.7), 2.69–2.79 (2H, m), 3.32 (1H, s), 4.03 (1H, dd, J = 8.8, 6.4), 4.10 (1H, qu, J = 7.0), 4.27 (1H, s), 4.34–4.40 (1H, m), 5.04 (2H, s), 6.92–6.96 (1H, br), 7.05–7.34 (12H, m); ^{13}C NMR (100 MHz, DMSO-d_6, 80°C) δ 17.3, 17.6, 18.7, 29.7, 38.0, 49.9, 50.4, 57.8, 65.1, 72.5, 125.1, 127.0, 127.2, 127.3, 127.8, 128.6, 136.6, 138.4, 155.1, 169.8, 171.6; HRMS (FAB+), calculated for MCs^+ $C_{50}H_{64}N_6O_{10}Cs$ m/z 1041.3738, found m/z 1041.3780.

Compound 13. Compound **5** (50 mg, 0.093 mmol) was converted to compound **8** (52 mg, 54%) as a white solid. Compound **13** (22 mg, 65%) was prepared from compound **8** (35 mg, 0.034)as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6, 80°C) δ 0.73 (6H, d, J = 6.8), 0.87 (3H, d, J = 6.5), 0.89 (3H, d, J = 6.5), 1.48 (2H, t, J = 6.8), 1.59–1.67 (1H, m), 1.88 (1H, se, J = 6.7), 2.70–2.80 (2H, m), 3.34 (1H, s), 4.04–4.93 (2H, m), 4.23 (1H, s), 4.32–4.38 (1H, m), 5.05 (2H, s), 7.02–7.36 (1^3H, m); ^{13}C NMR (100 MHz, DMSO-d_6, 80°C) δ 17.2, 18.7, 21.1, 22.3, 23.8, 29.8, 37.9, 40.3, 50.4, 53.2, 57.4, 65.1, 72.2, 125.1, 126.9, 127.1, 127.2, 127.7, 128.5, 136.5, 138.3, 155.3, 169.7, 171.3; HRMS (FAB+), calculated for MCs^+ $C_{56}H_{76}N_6O_{10}Cs$ m/z 1125.4677, found m/z 1125.4720.

Compound 14. Compound **5** (49 mg, 0.091 mmol) was converted to compound **9** (68 mg, 68%) as a white solid.

Compound **9** (43 mg, 0.039) was then deprotected to give compound **14** (30 mg, 72%) as a white solid: ^{1}H NMR (400 MHz, DMSO-d_6, 80°C) δ 0.73 (6H, d, J = 6.8), 1.88 (1H, se, J = 6.6), 2.70–2.81 (4H, m), 3.36 (1H, s), 4.09 (1H, dd, J = 8.6, 6.4), 4.28–4.42 (3H, m), 4.95 (2H, s), 7.04–7.08 (2H, m), 7.12–7.32 (15H, m), 7.40–7.43 (1H, m); ^{13}C NMR (100 MHz, DMSO-d_6, 80°C) δ 17.3, 18.7, 29.8, 37.0, 37.9, 50.5, 55.7, 57.7, 65.0, 72.3, 125.1, 125.6, 126.8, 127.1, 127.3, 127.5, 127.7, 128.5, 128.6, 137.6, 137.9, 138.3, 155.4, 169.8, 170.6; HRMS (FAB+), calculated for MCs^+ $C_{62}H_{72}N_6O_{10}Cs$ m/z 1193.4364, found m/z 1193.4323.

Biological Assays. Kinetic determinations for both HIV and FIV PRs were performed at 37°C at pH 5.25 in duplicate by using F-2000 fluorescence spectrophotometer (Hitachi).

For HIV PR, the K_M and V_{max} values for the fluorogenic peptide substrate 2-aminobenzoyl (Abz)-Thr-Ile-Nle~Phe-(*p*-NO_2)-Gln-Arg-NH_2 (23) were determined by measuring the initial rate of hydrolysis at different substrate concentrations (5.0, 7.5, 10, 20, 35, 50, 100, and 200 μM) by monitoring the change in fluorescence at an excitation wavelength of 325 nm and an emission wavelength of 420 nm, and fitting the obtained data to the Michaelis–Menten equation by using the GRAFIT program (version 3.0, Erithacus Software, U.K.). Assays were run in 0.1 M Mes buffer, containing 5% (vol/vol) glycerol and 5% (vol/vol)

DMSO (200 μl final volume). The enzyme concentration (30 μg/ml) that gave ideal progress curve was used for assays, but the dimeric active HIV PR concentration was not accurately determined. The K_i for each inhibitor of HIV PR was determined by obtaining the progress curve with the inhibitor (2.0–9.0 nM) at different substrate concentrations (7.5, 10, 20, 35, and 50 μM) under the same reaction conditions as above. The curve fit of the data was determined, and the subsequent K_i was derived by using the GRAFIT program.

For FIV PRs, the kinetic data were determined under reaction conditions similar to those for HIV PR. The K_M and V_{max} for the fluorogenic substrate Arg-Ala-Leu-Thr-Lys(Abz)-Val-Gln~nPhe-Val-Gln-Ser-Lys-Gly-Arg were determined by monitoring the change in fluorescence at an excitation filter of 325 nm and an emission filter of 410 nm with the GRAFIT program under the following reaction conditions: substrate concentration (6.0, 10, 20, 35, 50, 100, and 200 μM), 0.1 M NaH_2PO_4 buffer containing 0.1 M Na citrate, 0.2 M NaCl, 1.0 mM DTT, 5% (vol/vol) glycerol, 5% (vol/vol) DMSO, and 7.5 μg/ml [FIV(3X) and FIV(V59I)] or 2.5 μg/ml [FIV(Q99V)] of the enzyme. The K_i for each inhibitor of FIV PRs was also determined by obtaining the progress curve with the inhibitor (50 nM–20 μM) at different substrate concentrations (10, 20, 35, and 50 μM). The progress of hydrolysis of each assay was recorded, and the inhibition constants were determined (Table 1).

Protease Constructs. *Autoproteolysis-resistant FIV PR.* FIV(3X) was constructed as described (24) and contains the G5I, N55T, and C84K codon mutations that block three primary autoproteolysis sites in the FIV PR. All clones were sequenced to confirm the modifications made to the FIV PR ORF. Kinetic analyses revealed no significant change in K_M or k_{cat} values between the autoproteolysis-resistant 3X PR and wild-type FIV PR (24).

Mutant FIV PRs. Mutant FIV PRs were prepared that contained substitutions of HIV residues noted to be associated with drug resistance in HIV (16) at equivalent sites in the three-dimensional structure.

FIV(Q99V). The FIV 34TF10 infectious molecular clone (FIV-34TF10) (12) was used as the template in a PCR by using a negative strand primer (5′-ATCTCTCCCCAATAATGGTAC-TATTAATGAGTTATCTTCT AAGAC-3′; complementary to nucleotides 2252–2297) that mutated the FIV PR Gln-99 codon to Val and the positive strand primer (5′-ACTATTGGACAT-ATGGCATATAATAAAGTAGG TACTACTAC-3′; nucleotides 1964–2005) that, when incorporated into the PCR product, added an initiation Met and Ala codon to the determined 5′ Tyr codon of the FIV/PR ORF (19) as well as a 5′ *Nde*I restriction site. The ≈300-bp PCR product was purified and used in a second PCR with the same template and with a negative strand primer (5′-ATCAGAAAGCTTTTACATTACTAACCTGATATTA-AATTT-3′; complementary to nucleotides 2306–2345) that added a stop codon after the determined C-terminal Met codon of the PR ORF (19) in addition to a 3′ *Hin*dIII restriction site, to facilitate cloning. The resulting PCR product was digested with *Nde*I and *Hin*dIII and ligated into pT7–7 (25), which had been digested with *Nde*I and *Hin*dIII, to give FIV(Q99V).

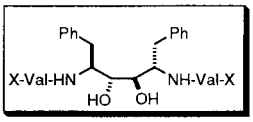
FIV(V59I). FIV-34TF10 was the template in a PCR with the positive strand primer (5′-GGAAGGCAAAATATGATTG-GAATTGGAGGAGGAAAGAGAGGAACA-3′; nucleotides 2135–2178) that mutates the FIV PR Val codon 59 to Ile, and the second negative strand primer used for FIV(Q99V). The ≈200-bp PCR product was purified and used in a second PCR with the same template and the positive strand primer used for FIV(Q99V). The resulting PCR product was digested with *Nde*I and *Hin*dIII and ligated into pT7–7 (25), which had been digested with *Nde*I and *Hin*dIII, to give FIV(V59I).

FIV Protease Expression and Purification. All PR expression constructs were transformed into the *Escherichia coli* cell line BL21(DE3) (26), which contains the T7 polymerase gene under control of the Lac promoter. Cultures were induced at OD_{600} = 0.5 with 1 mM IPTG for 5 hr with the PR inclusion bodies isolated and then solubilized in 8 M urea/10 mM Tris, pH 8.0/5 mM EDTA. The PRs were subsequently purified and renatured as described (24) and either stored at −70°C or brought to 50% glycerol and stored at −20°C.

HIV PR Expression and Purification. A recombinant plasmid bearing a portion of the Pol gene of the BH10 clone of HIV was used for amplification of sequence encoding PR. The 5′ primer was constructed so as to insert an initiator methionine as part of the coding sequence for an *Nde*I site, eight amino acids before the beginning of PR. This primer also encoded a nucleotide change to mutate Gln-7 to Lys, to block a major site of autoproteolysis (27), and thus increase stability of the enzyme. The 3′ primer was designed to insert a stop codon immediately after residue 99 of PR, and a *Hin*dIII site 3′ of the stop codon, to facilitate directional cloning. The PCR product was then cut with *Nde*I and *Hin*dIII and inserted into the pET 21a+ vector (Novagen) for protein expression.

The recombinant plasmid was transformed into the BL21.DE3, p lys S strain of *E. coli* (26). Inclusion bodies were prepared and solubilized essentially as described for preparation of FIV PR (24). The washed inclusion body pellet was then solubilized in 200 ml 20 mM Tris·HCl, pH 8/1 mM DTT/5 mM EDTA/8 M urea with stirring at 4°C for 1 hr. Insoluble material was removed by centrifugation at 8,000 × *g* for 30 min. The supernatant from this centrifugation was treated batchwise by the addition of 20 gm DE 52 anion-exchange resin, and the mixture was stirred at 4°C for 1 hr. After centrifugation, PR

Table 1. Inhibition of FIV and HIV PRs by C_2-symmetric diols



Inhibitor (X)	FIV (3X)* K_i, nM	FIV (V59I)* K_i, nM	FIV (Q99V)* K_i, nM	HIV (wt)† K_i, nM
10 (Cbz)	17,000 ± 2,000	17,300 ± 2,200	16,000 ± 2,000	1.1 ± 0.2 (<1.0)‡
11 (Gly-Cbz)	156 ± 18	101 ± 17	37 ± 9	1.7 ± 0.3
12 (Ala-Cbz)	41 ± 7	22 ± 5	8.3 ± 1.3	1.5 ± 0.3
13 (Leu-Cbz)	159 ± 15	ND	ND	1.4 ± 0.3
14 (Phe-Cbz)	7,000 ± 500	6,800 ± 1,300	3,700 ± 600	2.6 ± 0.4

K_i values were determined in duplicate. ND, not determined. wt, wild type.
*Data obtained at pH 5.25 at 37°C in 0.1 M NaH_2PO_4, 0.1 M sodium citrate, 0.2 M NaCl, 0.1 mM DTT, 5% glycerol, and 5% DMSO in volume.
†Data obtained at pH 5.25 at 37°C in 0.1 M Mes 5% glycerol and 5% DMSO in volume.
‡Data obtained (5) at pH 5.5.

was found in the supernatant. The resin was washed once with 50 ml of resuspension buffer (above), and the wash and supernatant fractions were combined.

The supernatant/wash fraction was then passed over Resource Q anion exchange resin equilibrated in resuspension buffer, by using a Pharmacia FPLC apparatus. The fraction that failed to bind to the column was concentrated by using 5 K cutoff UltraFree centrifugal concentrators (Millipore). The retentate was then dialyzed overnight against deionized water, which caused precipitation of PR. The pellet was recovered by centrifugation at 3,000 $\times$ *g* for 20 min, then resuspended in 20 mM sodium acetate, pH 5.3/1 mM DTT/5 M GuHCl to a concentration of 1 mg/ml (determined by Lowry assay of the pellet suspended in a known volume of water before final pelleting and solubilization in sodium acetate/DTT/GuHCl buffer). Matrix-assisted laser desorption ionization analysis indicated a mass of 10,792, which is within 1 mass unit of the predicted mass for the properly processed PR. Activity was monitored by using a fluorogenic substrate, as detailed above. Aliquots were stored at -70°C for subsequent use.

***Ex Vivo* Inhibitor Analyses Against FIV.** The lymphocytic cell line 104-C1 (provided by C. Grant, Custom Monoclonals) was used as the target for infection. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 200 μM L-glutamine, 1$\times$ MEM-vitamins, 100 μM sodium pyruvate, 1$\times$ nonessential amino acids, 5.5×10^{-5} M 2-mercaptoethanol, 50 μg/ml gentamicin, 50 units/ml human recombinant interleukin-2 (provided by Hoffmann–LaRoche), and 7.5 μg/ml concanavalin-A. For inhibitor assessment, 5×10^6 cells were infected with FIV-PPR (4×10^5 RT units) in 1 ml culture for 2 hr at 37°C. No-virus and virus-only controls were incubated in a similar manner. The cells were then washed with Hanks'-buffered saline solution and resuspended in 10 ml of complete medium. Compound **12** (10 mg/ml in DMSO) was then added to final concentrations of 0.1, 0.5, 1, and 5 μg/ml in duplicate. The cells were then incubated at 37°C. The culture supernatants were monitored for the presence of pelletable reverse transcriptase activity at weekly intervals, as follows. Cell-free culture supernatants (4 ml) were centrifuged at 60 K for 30 min, and the pellets were resuspended in 100 μl of lysis buffer containing 40 mM Tris·HCl (pH 8.1), 360 mM NaCl, 20 mM DTT, and 0.2% Nonidet P-40. Twenty-five microliters of lysate was added to 25 μl of a mixture containing poly(rA-pdT) (Pharmacia), $MgCl_2$, and ^{3}H-labeled deoxythymidine 5′-triphosphate (DuPont/NEN) and incubated for 1 hr at 37°C. The mixture was spotted on DE81 paper, air-dried, fixed in 0.1 M sodium pyrophosphate, and washed three times in 0.3 M ammonium formate and an additional time in 95% ethanol. The paper was then dried and counted on a scintillation counter.

Cells were split 1:5 after the second, third, and fourth time points, and fresh inhibitor was added at the appropriate concentration at each of these intervals. The data were expressed as values $+/-$ standard deviation of the mean (Fig. 2*A*).

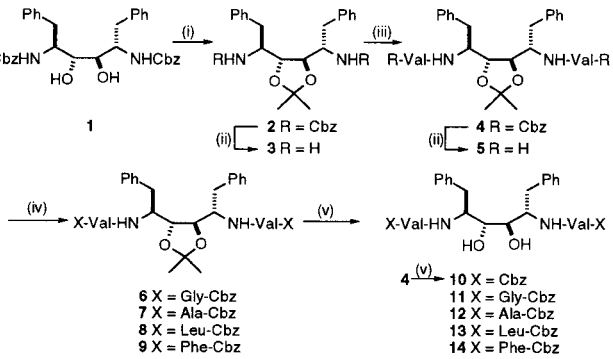
***Ex Vivo* Inhibitor Analyses Against HIV.** WEAU-1.6 [a kind gift of George Shaw, University of Alabama (28)], a CXCR4 utilizing isolate, was used for all studies. MT-2 cells (2×10^5) in 1 ml were infected for 4 hr, at 37°C, by using WEAU-1.6 at 25 $TCID_{50}$. Before establishing cultures for inhibitor assessment, infected cells were washed three times with complete medium (CM), which was RPMI 1640 supplemented with 10% FBS, 100 mM sodium pyruvate, 200 mM L-glutamine, and 50 mg/ml of gentamicin sulfate, to remove any unbound virus. For inhibitor assessment, cultures were established in duplicate or triplicate by using 5×10^4 infected cells and 1×10^5 uninfected cells in a total volume of 1.5 ml in Costar 6-well plates. Cultures were split 1:4 every 3 days and given fresh CM. Compound **12** (from a 10 mg/ml stock in DMSO) was added at 1 or 5 μg/ml at initiation of the culture and after each split. Testing of the antiviral efficacy of compound **12** in MT-2 was assessed every day by using an Olympus (model) inverted microscope equipped with phase-contrast objectives. When MT-2 cells are infected with WEAU-1.6, they form syncytia and die within 24 hr, events easily discernible visually. A total of 200 cells were counted in each well, and when syncytia were noted, cells were removed and tested for viability by using trypan blue. Results are expressed as the average of viable cells.

***Ex Vivo* Inhibitor Analyses Against SIV.** Stocks of SIVmac251 (provided by R. Desrosiers, Harvard University) were prepared in 174xCEM cells (provided by the National Institutes of Health AIDS Research and Reference Program) grown in 88% RPMI medium containing 20 mM Hepes and 12% heat-inactivated fetal calf serum. A 24-hr supernatant was collected at day 14 postinfection and aliquoted and stored at -80°C for subsequent experiments.

Cells were acutely infected with approximately 400 $TCID_{50}$ units of SIVmac251 for 90 min at 37°C. The cells were collected by centrifugation, washed twice with medium to remove free virus, then plated in 0.45 ml medium in 48-well tissue culture plates, at 10^5 cells per well. Compound **12**, prepared as a 10 mg/ml stock in DMSO, was then added to final concentrations of 10, 1.0, 0.1, and 0.001 μg/ml, in triplicate cultures. Triplicate control cultures received medium only. Uninfected cells were also cultured with the above concentrations of compound **12**, with no effects noted. Wells were observed for the presence of syncytia at 72 and 96 hr postinfection, and supernatants collected at 96 hr were assayed for relative amounts of p27 antigen by using a quantitative ELISA assay (Coulter).

RESULTS

Chemical Syntheses. The (1*S*, 2*R*, 3*R*, 4*S*)-1,4-bis[(*N*-Cbz)amino]-1,4-dibenzyl-2,3-diol **1** was prepared by Pedersen's procedure (22) along with its diastereomers. After protection of the diol as the isopropylidene **2**, these minor diastereomeric impurities were removed by flash-column chromatography. The Cbz groups of compound **2** were deprotected by hydrogenation, then diamine **3** was coupled with Cbz-Val by using HBTU (29), providing adduct **4**. Four different P3 and P3′ residues were then introduced to adduct **4** by applying the same deprotection and coupling procedures described above to give compounds **6-9**. Finally, the target inhibitors **11–14** were obtained by removal of the isopropylidene from the corresponding precursor under acidic conditions. The same procedure was applied in the synthesis of other compounds (~15), which were not shown here. The reference inhibitor **10** was also synthesized from compound **4** by the same deprotection procedure (Scheme 1).



(i) 2,2-dimethoxypropane, *p*-TsOH; (ii) Pd/C, H_2, MeOH; (iii) HBTU, Cbz-Val, Et_3N,CH_3CN; (iv) HBTU, Cbz-amino acids, Et_3N, CH_3CN; (v) *p*-TsOH, MeOH.

SCHEME 1. Synthesis of C_2-symmetric inhibitor.

***In Vitro* Inhibitory Activities Against Proteases.** The inhibitory effects of each inhibitor were evaluated against HIV and FIV PRs along with two mutant FIV PRs that contain amino

acid substitutions corresponding to those in HIV PR at homologous sites. The results of some selective inhibitors, each of which is a competitive inhibitor of all four enzymes, are summarized in Table 1.

All the C_2-symmetric diols tested in this study showed very high potency against HIV PR, and their K_i values ranged between 1.1 and 2.6 nM. Considering experimental error, there was no significant difference in the overall efficacy of these diols in inhibition of HIV PR. In part, this reflects the low restriction of amino acid residues at the S3 and S3′ subsites of HIV PR. In addition, the Cbz groups of the reference inhibitor **10**, which does not contain P3 and P3′ residues, could be positioned tightly at the S3 and S3′ subsites of HIV PR to make compound **10** an effective inhibitor. However, inhibition of FIV PR by inhibitors **10-14** showed a remarkably different pattern. First, the inhibitory activity of the reference compound **10** was decreased by almost 1.7×10^4-fold compared with its K_i for HIV PR. This striking activity loss observed for **10** was recovered by extending the backbone of the inhibitor by using Gly as P3 and P3′ residues, with the K_i of **11** being 110-fold lower than **10**. This preference of the extended inhibitor backbone found in FIV PR is also supported by the observation that HIV PR will cleave a six-residue peptide substrate, Ac-Gln-Ala-Tyr⌣Pro-Ile-Gln, whereas the smallest FIV PR substrate that can be efficiently cleaved is an eight-residue peptide, Ac-Pro-Gln-Ala-Tyr⌣Pro-Ile-Gln-Thr (30). The best residues for S3 and S3′ binding are Ala and Ser. In fact, inhibitor **12** (K_i = 41 nM) is the most potent inhibitor of FIV PR known to date. The inhibitory activity of **12** against FIV PR was reduced by increasing the size of the side chain of the P3 and P3′ residues, with the K_i of **13** 4-fold higher than **12**. Furthermore, the diol **14** showed 45- and 170-fold lower potency compared with **11** and **12**, respectively, and this result suggests that the benzyl side chain of P3 and P3′ residues may cause unfavorable interaction with FIV PR or the neighboring P1 and P1′ side chains. This severe restriction of P3 and P3′ moieties in FIV PR partly explains the total loss of potency against FIV PR by HIV PR inhibitor Ro 31–8959, because it contains bulky aromatic group at the P3 position.

The result was further confirmed by comparison of the x-ray structures of FIV (15) and HIV PRs (21) complexed with inhibitors. The structures revealed that only two residues (Arg-13, Asp-34) in the S3 and S3′ subsites of FIV PR were conserved at the structurally aligned HIV PR positions (15, 21). Three other residues at the subsites Gly-48, Pro-81, and Val-82 of HIV PR were replaced with Ile-57, Ile-98, and Gln-99 in FIV PR (15, 21). As a result, the S3 and S3′ subsites of FIV PR are sterically more congested than those in HIV PR, and these three different residues may define the S3 and S3′ subsite specificities of the enzymes (15). In addition, the Gly-48 and Val-82 of HIV PR have been identified as frequently mutated residues to develop drug resistance (31–34). For example, the potency of the FDA-approved drugs containing bulky P3 moieties Ro 31–8959 and ABT-538 against G48V mutant was decreased by 27- and 17-fold, respectively (31). Among the HIV PR variants containing mutations at Val-82 (31–34), the V82F mutant becomes 15-, 7-, and 90-fold less sensitive toward the drugs AG-1343, MK-639, and ABT-538, respectively (31, 33). Weak inhibition observed in mutant HIV PRs with inhibitors containing large P3 and P3′ moieties are consistent with binding preferences found in FIV PR. These observations further support utility of FIV PR as a model for drug-resistant variants.

The results from the inhibition studies of the diols **10–12** and **14** against the mutant FIV PRs are also intriguing. The less effective inhibitors **10** and **14** showed very similar activities against mutants compared with wild-type FIV PR. However, the efficacy of inhibition by the more potent inhibitors **11** and **12** was progressively increased up to 5-fold in mutant enzymes. Overall, these results provide new insights into the specificity (35) and resistance development (36, 37) of the aspartyl proteases and may help development of new inhibitors that are better than those currently available (1–9).

***Ex Vivo* Inhibitory Activities of Compound 12.** The ability of the most potent FIV PR inhibitor **12** to prevent infection of FIV, HIV, and SIV in tissue culture was examined. The results are summarized in Fig. 2.

For FIV, the assays were performed in FIV-infected feline T cells that were cultured in the presence of compound **12** at different concentrations over the course of 1 month. Each data point in Fig. 2*A* represents the amount of pelletable FIV reverse transcriptase in the culture supernatant. Compound **12** was able to markedly inhibit FIV replication at 0.5 μg/ml (0.55 μM) and found to be most effective at 1.0 μg/ml (1.1 μM). Furthermore, this inhibitor was not toxic to feline T cells. After 1 month, the drug-treated cultures were split and replated with and without compound **12**. No virus was detected in the absence or presence of drug after 24 weeks in culture (not shown). No sign of resistance development against the drug has been observed after 8 weeks of continuous culture.

The results from tissue culture assays against SIV (Fig. 2*B*) and HIV (Fig. 2*C*) were equally encouraging. Compound **12**, at 1 μg/ml, reduced SIV virus expression levels to near background, as judged by reduction in release of p27 antigen into the culture supernatant in the presence of drug (Fig. 2*B*). The effectiveness of compound **12** against HIV was measured by determining the

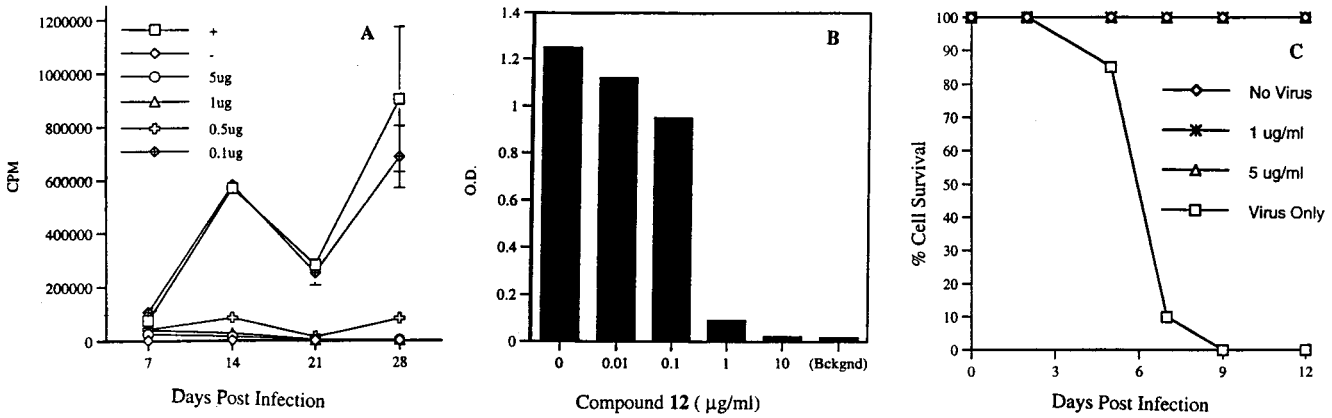


FIG. 2. Inhibitory activities of compound **12** against FIV (*A*), SIV (*B*), and HIV (*C*) in tissue culture. (*A*) Amount of FIV was determined by counting pelletable FIV reverse transcriptase in culture supernatant. (*B*) The effectiveness of the inhibitor against SIV was determined by using a quantitative ELISA assay by measuring OD at 405 nm at 4 days postinfection. (*C*) The level of efficacy of inhibition of HIV replication was assessed by measuring viable cells in cultures. Against all three virus, compound **12** showed almost the same degree of potency in tissue cultures and became most effective at 1.0 μg/ml (1.1 μM) to block nearly 100% of viral reproduction.

percentage of viable cells remaining in culture over time in the presence and absence of drug (Fig. 2*C*). In the control, HIV caused formation of multinucleated syncytia and 100% cell death by 9 days postinfection. However, the cells cultured with 1 μg/ml (1.1 μM) or 5 μg/ml (5.5 μM) of inhibitor **12** remained 100% viable after 1 month, which is identical to results obtained in the absence of virus infection. To test for virus in these cultures, supernatants were removed and added to 1×10^5 uninfected MT-2 cells after a 1:5 dilution with fresh CM. After 3 weeks the MT-2 cells remained uninfected, demonstrating the absence of free virus in cultures of infected MT-2 cells treated with compound **12**. In contrast, when 2×10^5 infected MT-2 cells that had been treated with compound **12** for 2 weeks were removed and replated in fresh medium with or without compound **12**, only MT-2 cells cultured in the absence of compound **12** were dead within 4 days (data not shown). These results clearly demonstrated strong potency of the drug against HIV as well as minimal toxicity to host cells. Cultures are being carried continuously in the presence of compound **12** to look for resistance development. Tests are also underway to determine the level of efficacy of the compound against a defined panel of drug-resistant HIVs; the preliminary results are promising and will be published separately.

DISCUSSION

It is clear that FIV PR exhibits a specific preference for amino acids containing small side chains at the P3 and P3′ positions, especially for Ala. In addition, extension of inhibitor backbone can increase the potency of inhibitors in FIV PR. Our *in vitro* inhibition studies of mutant FIV PR also showed a direct relationship between the inhibition of FIV PR and HIV PR. This observation suggests that potent inhibitors of FIV PR, containing P3 to P3′ residues, become even more efficient against HIV PR.

The most potent inhibitor **12** has also shown strong ability to control lentiviral infections in tissue culture. In fact, this compound inhibits replication of FIV, HIV, and SIV with virtually the same degree of effectiveness. This remarkable versatility of compound **12** also suggests the strong possibility of sustaining its potency against mutant HIV PRs. Experiments are underway to test this hypothesis. Finally, although the current results represent only an initial step toward developing potential therapeutic agents against HIV PR as well as mutant enzymes, using the FIV system for advancing HIV therapies is clearly an effective strategy where target drugs have dual efficacy against FIV and HIV. As a natural animal system, FIV offers the ability to perform *in vivo* tests of efficacy and assessment of drug resistance that is not readily feasible in primate systems. In addition, it is hoped that the broad-based nature of inhibitors arising from these studies will afford a reduced level of resistance development.

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