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POTENT INHIBITORS OF THE HIV-1 PROTEASE WITH GOOD ORAL BIOAVAILABILITIES

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Summary. A series of novel pseudo-symmetrical and unsymmetrical inhibitors based on the backbone modification of a peptidomimetic were synthesized and found to be highly potent inhibitors of the HIV-1 protease (IC_{50} = 2.9 to <0.5 nM). These compounds also possess good antiviral activity *in vitro* as measured by inhibition of the cytopathic effect of HIV-1_{3B} in MT-4 lymphocytes. Importantly, some of these compounds also have good oral bioavailabilities in rats (F = 30.6% to 100%). One of these compounds **4C**, also has good oral bioavailability in beagle dogs and cynomolgus monkeys.

The widespread occurence and extremely unfavorable prognosis of human immunodeficiency virus (HIV) infection makes the discovery of new and effective therapy extremely urgent. Of the currently licensed therapeutic agents which target the HIV reverse transcriptase, mostly have undesirable toxicities and select resistant mutant virus strains in a relatively short time (1). The HIV protease is an essential viral enzyme which proteolytically processes the gag and gag-pol polyproteins to form the mature proteins needed for the production of infectious viral particles (2). The HIV-1 protease is a member of the aspartic acid family and has been shown by X-ray crystallography to be a C-2 symmetrical dimer (3,4). Viral progeny that lack a functional protease are not infectious (5). For this reason, the HIV protease is considered as an important target for the development of agents for the treatment of HIV infection. Towards this goal, synthetic inhibitors of HIV protease have been shown to inhibit the spread of HIV infection *in vitro* (6,7). Unfortunately, most of the reported compounds are peptidomimetics that lack significant oral bioavailability (8), which is an important obstacle for their development as useful therapeutic agents. However,

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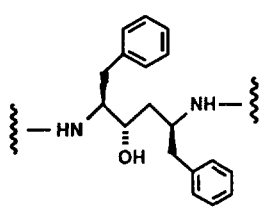
we have recently identified a clinical candidate, ABT-538, which exhibits potent antiviral activity and excellent oral bioavailability (9) (see Fig 1).

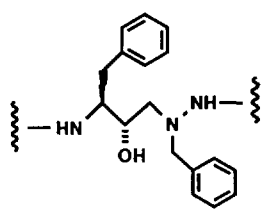
We now wish to report the efficient synthesis and biological evaluations of a novel series of HIV-1 protease inhibitors which contain a backbone modification of the pseudo-symmetric core unit of ABT-538. This series, which is represented by the generic structure **4** in Scheme 1, shows highly potent and selective activity against the HIV-1 protease. The activities of these compounds against other aspartic acid proteases such as renin, pepsin or cathepsin D are at least five to six orders of magnitude less potent. They also possess good antiviral activities *in vitro* against the spread of HIV-1 infection by MT-4 cell cultures (cytopathic effect). The hydrolyzable amide bond of the -Phe (P_1) -Pro (P_1')- cleavage site of the substrate of the HIV protease is replaced by a non-hydrolyzable hydroxyethylene unit and the α-carbon of the P_1' side chain was replaced by a nitrogen. As shown in Scheme 1, the P_1' side chain can be readily changed to a wide variety of substituents (R_2 in Table 1) simply by variation of the hydrazines used in the opening of the epoxide. Also, since the intermediate **2** is orthogonally protected, the left or right side of the molecule can be selectively deprotected at either end and coupled to other fragments. Importantly, several of the compounds in this series also have good oral bioavailabilities in three different species (rats, dogs or cynomolgus monkeys).

Materials and Methods

Synthesis. The synthesis of compounds represented by the generic structure **4** is outlined in Scheme 1. The opening of the epoxide **1** with substituted hydrazines was accomplished by refluxing in isopropanol (10). The benzyloxycarbonyl protecting group was removed under hydrogenolysis conditions and the carbamate group with different R_1 was synthesized by coupling with the appropriate carbonate derived from p-nitro-phenyl chloroformate and R_1-OH. Removal of the t-butyloxycarbonyl protecting group under acidic conditions (trifluoroacetic acid and dichloromethane) and coupling to L-valine (protected at the N-terminal as a N-methyl urea or carbamate) using DCC/HOBt coupling conditions provided compound **4**.

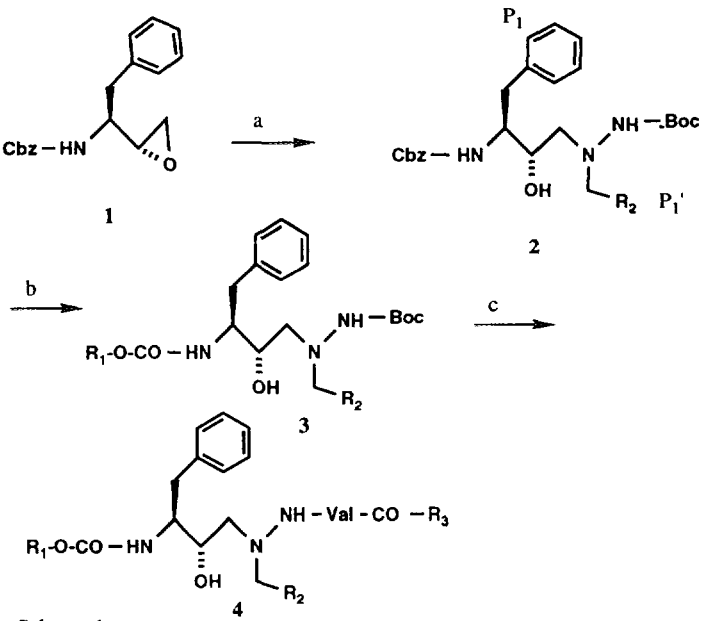


core-unit of ABT-538



aza backbone modified core-unit

Figure 1



Scheme 1.
Reagents and conditions: (a) Boc-NH-NH-CH_2-R_2/ isopropanol; (b) i, H_2/ Pd/C; ii, R_1-O-CO-p-nitrophenyl; (c) i, TFA/CH_2Cl_2; ii, DCC/ HOBt and R_3CO-Val-OH.

Protease Inhibition Assay. HIV-1 protease inhibition was measured by a fluorescence assay (11) using recombinant HIV-1 protease, isolated as described previously (12). Reactions were carried out at 30°C in reaction mixtures containing the following in a final volume of 300 µl: 125 mM sodium acetate, pH 4.5; 1 M sodium chloride; 0.5 mM dithiothreitol; 0.5 mg/ml bovine serum albumin; 1.3 µM fluorogenic substrate (20). The fluorogenic substrate used is Dabcyl-Ser-Gln-Asp-Tyr-Pro-Ile-Val-Gln-EDANS wherein DABCYL = 4-(4-*dimethylaminophenyl)azobenzoic acid* and EDANS = 5-((2-*aminoethyl)amino)*-naphthalene-1-sulfonic acid. Inhibitors, when present, were added from stock solutions made in dimethylsulfoxide. The final dimethylsulfoxide concentration was adjusted to 2% in all cases. The reactions were initiated by the addition of approximately 1 nM HIV-1 protease and rates were determined by following the change in fluorescence intensity (excitation 340 nM, emission 490 nM) that accompanies the cleavage of the fluorogenic substrate. The percent inhibition of enzyme activity in the presence of inhibitor was determined by comparing inhibition rates with uninhibited control reactions. All IC_{50} values were determined with a range of inhibitor concentrations using the relation IC_{50} = (100 ÷ percent inhibition - 1) x inhibitor concentration. IC_{50} values should closely approximate true Ki values since the substrate concentration is much less than the Km determined previously (12). For compounds with IC_{50} value less than 0.5 nM, their potency in the enzyme inhibition assay are shown as percent inhibition at 0.5 nM in Table 2.

Antiviral Assays. The *in vitro* antiviral activities of the compounds are determined by the inhibition of the cytopathic effect (CPE) of HIV-1_{3B} in MT-4 cells (13). MT-4 cells (1×10^4) were infected with 10 $TCID_{50}$ of HIV-1_{3B} for 1 hour, washed, and cultured at 37°C in RPMI 1640 + 10% FBS medium containing inhibitors (fluoroketones) in triplicate. Cytopathic effect and toxicity were monitored at day 5 by MTT uptake in 96-well microtiter plates using a Biotek model EL 320 at a wavelength of 570-650 nM. The EC_{50} values are shown in Table 2.

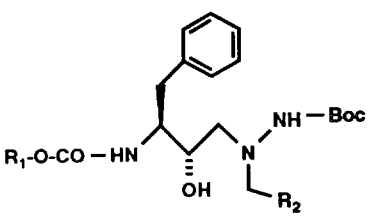
Pharmacokinetic Analysis. Sprague-Dawley derived rats (male, 0.25-0.35 Kg), beagle dogs (male/female, 8-12 Kg) and cynomolgus monkeys (female, 2.6-4 Kg) received a 5 mg/kg i.v. bolus (over about 60 s), or a 5 mg/kg or 10 mg/kg oral gavage. Plasma samples, obtained as a function of time after dosing, were analyzed by reverse-phase HPLC as described previously (14).

Results and Discussion

As shown in Table 1, a series of compounds with generic structure **3** was readily synthesized, incorporating a wide variety of P_1' substituents. Compound **2a**,

Table 1

Pseudo-symmetrical Inhibitors of HIV-1 Protease



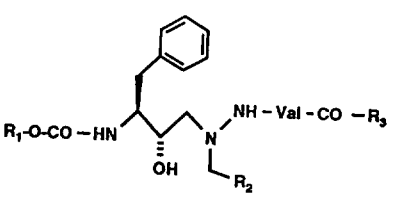
Number	R_1	R_2	IC_{50} nM
3a	benzyl	phenyl	5.1
3b	3-pyridylmethyl	phenyl	3.9
3c	5-thiazolylmethyl	phenyl	4.4
3d	benzyl	4-methoxyphenyl	10
3e	benzyl	3-methoxyphenyl	13
3f	benzyl	3-furanyl	3.8
3g	benzyl	3-furanyl	3.9
3h	benzyl	4-fluorophenyl	13
3i	benzyl	4-chlorophenyl	16
3j	benzyl	4-hydroxyphenyl	7.5
3k	benzyl	3-thiazolyl	35
3l	benzyl	5-oxazolyl	16
3m	benzyl	4-isoxazolyl	11
3n	benzyl	4-pyridinyl	104
3p	benzyl	cyclohexyl	38
3q	benzyl	4-pyranyl	36

with a phenyl substituent, is a potent inhibitor of the HIV-1 protease (IC_{50} = 5.1 nM). Varying the R_1 group at the left N-terminus from benzyl to pyridyl or thiazole in **3b,3c** do not affect the enzyme inhibition activity. Other heterocycles such as furan, thiazole, oxazole, isoxazole or substituted phenyls (3-methoxy, 4-fluoro, 4-chloro) can be substituted for the P_1' phenyl without significant loss of activity. This shows that the P_1' pocket of the enzyme can tolerate quite a wide range of aromatic heterocycles. On the other hand, substitution of 4-pyridyl resulted in a 20x loss of activity. Saturation of the phenyl ring to cyclohexyl or 4-pyranyl also is not favorable (~10x loss in activity).

Insertion of a N-protected valine to the right side N-terminus resulted in another series of unsymmetrical inhibitors as shown in Table 2. All of these compounds (except **4e**) have IC_{50} of 0.5 nM or less. Adding one amino acid residue increases the protease inhibition activities at least 10-20x. These compounds also exhibited good antiviral activities by inhibition of the cytopathic effect of HIV-1 in MT-4 cells as shown in Table 2. There may seem to be a 1000x difference in the IC_{50} and EC_{50} values. The IC_{50} measures the inhibitory potency of the compounds against purified enzymes in a test tube, while the EC_{50} measures the inhibitory potency of the compounds against the spread of the HIV-1 infection in a cell culture and requires the compounds to penetrate the cell membranes. Other factors such as solubilities of the compounds in the culture medium may affect their EC_{50} values.

Table 2

Un-symmetrical Inhibitors of the HIV-1 Protease



Cmpd. No.	R_1	R_2	R_3	IC_{50} (nM)[a]	EC_{50} (μM)[b]
4a	3-pyridinyl-CH_2-	phenyl	3-pyridinyl-CH_2-(N-CH_3)	68% at 0.5 nM	0.16
4b	5-thiazolyl-CH_2-	phenyl	2-isopropyl-4-oxazolyl-CH_2-(NCH_3)	78% at 0.5 nM	0.11
4c	5-thiazolyl-CH_2-	phenyl	2-isopropyl-4-thiazolyl-CH_2-(NCH_3)	77% at 0.5 nM	0.10
4d	5-isoxazolyl-CH_2-	phenyl	2-isopropyl-4-oxazolyl-CH_2-(NCH_3)	71% at 0.5 nM	0.10
4e	5-thiazolyl-CH_2-	4-pyranyl	2-isopropyl-4-thiazolyl-CH_2-(NCH_3)	3.2	1.13
4f	5-thiazolyl-CH_2-	3-furanyl	2-isopropyl-4-oxazolyl-CH_2-(NCH_3)	75% at 0.5 nM	0.029
4g	5-thiazolyl-CH_2-	3-hydroxyphenyl	2-isopropyl-4-thiazolyl-CH_2-(NCH_3)	0.5	1.37
4h	5-thiazolyl-CH_2	3-tetrahydrofuranyl	2-isopropyl-4-thiazolyl-CH_2-(NCH_3)	67% at 0.5 nM	0.12
4i	5-thiazolyl-CH_2-	4-hydroxyphenyl	2-isopropyl-4-thiazolyl-CH_2-(NCH_3)	56% at 0.5 nM	0.04
ABT-538				71% at 0.5 nM	0.036

[a]Concentration of test compound at which 50% of the activity of purified, recombinant HIV-1 protease upon a fluorogenic substrate was inhibited, for compounds with IC_{50} less than 0.5nM, the potency is reported as % inhibition at 0.5nM.
[b]Concentration of compound which inhibited 50% of the cytopathic effect of HIV-1_{3B} in MT-4 lymphocytes *in vitro*. Values represent the result of a single triplicate assay, using a multiplicity of infection (MOI) of either 0.001 or 0.032 tissue culture infective dose per cell.

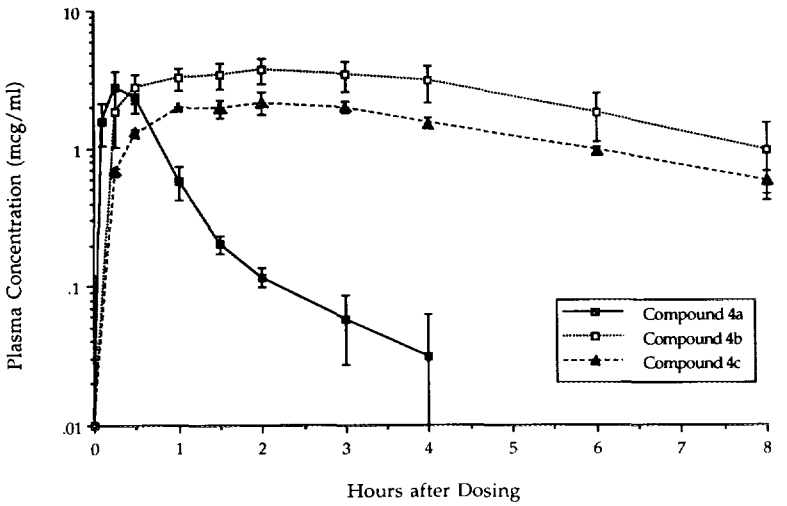


Figure 2.
Mean (±SEM) Plasma Concentrations of Parent Drug after a 10 mg/kg Oral Dose in Rat.

Addition of a valine residue to this series of compounds increases their peptidic nature and might impede their oral absorption(8). Compounds **4a**, **4b** and **4c**, which incorporate valine and a variety of N-terminal groups were found to exhibit oral bioavailabilities of 30.6%, ~100% and 82.6% respectively, in rats following a 10 mg/kg dose (Figure 2). The oral bioavailabilities of the compounds were determined by an identical HPLC assay as for ABT-538(9). Since it is not a bioassay, it does not measure the anti-HIV activity of the serum samples. Oral bioavailability (%F) does not necessarily coincide with anti-HIV activity because of other possible complicating factors such as protein binding in the serum.

Compound **4c** was tested for oral bioavailabilities in two other species - dogs and cynomolgus monkey. Compound **4c** has oral bioavailabilities of 49.5% in beagle dogs at dose of 5 mg/kg and 61.2% at dose of 5 mg/kg in cynomolgus monkeys. The series of compounds shown in Table 2, represents a novel series of compounds which demonstrated: high potency in the inhibition of the HIV-1 protease (IC_{50} < 0.5 nM); good antiviral activity *in vitro* against the cytopathic effect of HIV-1 virus in MT-4 cells (EC_{50} from 1.37 to 0.04 μM); and in selected cases, good oral bioavailabilities (15,16) in three different species. The investigation of these compounds as potential AIDS therapeutics may be warranted.

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