

Improved cyclic urea inhibitors of the HIV-1 protease: synthesis, potency, resistance profile, human pharmacokinetics and X-ray crystal structure of DMP 450

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Background: Effective HIV protease inhibitors must combine potency towards wild-type and mutant variants of HIV with oral bioavailability such that drug levels in relevant tissues continuously exceed that required for inhibition of virus replication. Computer-aided design led to the discovery of cyclic urea inhibitors of the HIV protease. We set out to improve the physical properties and oral bioavailability of these compounds.

Results: We have synthesized DMP 450 (bis-methanesulfonic acid salt), a water-soluble cyclic urea compound and a potent inhibitor of HIV replication in cell culture that also inhibits variants of HIV with single amino acid substitutions in the protease. DMP 450 is highly selective for HIV protease, consistent with displacement of the retrovirus-specific structural water molecule. Single doses of 10 mg kg⁻¹ DMP 450 result in plasma levels in man in excess of that required to inhibit wild-type and several mutant HIVs. A plasmid-based, *in vivo* assay model suggests that maintenance of plasma levels of DMP 450 near the antiviral IC₉₀ suppresses HIV protease activity in the animal. We did identify mutants that are resistant to DMP 450, however; multiple mutations within the protease gene caused a significant reduction in the antiviral response.

Conclusions: DMP 450 is a significant advance within the cyclic urea class of HIV protease inhibitors due to its exceptional oral bioavailability. The data presented here suggest that an optimal cyclic urea will provide clinical benefit in treating AIDS if it combines favorable pharmacokinetics with potent activity against not only single mutants of HIV, but also multiply-mutant variants.

Introduction

More is known about the structure [1–4] and essential functions [5–7] of the virally encoded protease of the human immunodeficiency virus (HIV) than perhaps any other single enzyme to date. The HIV protease has thus become an important target for the development of therapeutics for the treatment of AIDS [8,9]. Five peptide-like inhibitors of the HIV protease are currently in clinical trials [10–14]; FDA approval has recently been granted to the inhibitors Saquinavir, Ritonavir and Indinavir [10–12]. The long-term clinical efficacy of these compounds is still under investigation, but the emergence of resistant viruses appears to be a potential limitation for all of them [15–18]. Emergence of mutant HIV with decreased susceptibility to a protease inhibitor requires viral replication to continue in the presence of drug; it is therefore crucial to maintain the inhibitor at blood levels

sufficient to inhibit the replication of both wild-type and mutant HIV. Unfortunately, the peptide-like nature and size of many HIV protease inhibitors limit their oral bioavailability and half-life in man, making high blood levels difficult to achieve and sustain [19–21]. Moreover, many peptide-like agents bind to plasma proteins, limiting the effective concentration of free drug available to interact with the intracellular target sites [22]. The challenges for designing a superior, or second-generation, inhibitor of the HIV protease are thus substantial.

We previously described the use of structure-based drug design to produce a novel series of cyclic ureas, which are potent inhibitors of the HIV protease [23]. Cyclic ureas incorporate the hydrogen-bonding equivalents of an enzyme-bound water molecule into a low-molecular-weight, conformationally rigid, seven-membered ring

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system. This structure permits optimal interaction of substituents with corresponding S1, S1', S2 and S2' pockets in the active site of the HIV-protease dimer. On the basis of favorable pharmacokinetics in the rat and the dog [24], the cyclic urea DMP 323 was selected for development proceeding to a Phase I clinical trial in non-HIV infected male volunteers.

Although DMP 323 was a potent and selective inhibitor of the HIV protease [25], inhibited viral replication in a diverse array of laboratory isolates and cell types [26], and persistently inhibited viral polyprotein processing in chronically infected cells [27], its physicochemical properties, in particular poor solubility in both aqueous and lipid media, limited development of an oral formulation that could reproducibly achieve satisfactory blood levels in humans. Our subsequent drug-discovery efforts focused on the identification of cyclic ureas with both improved physical properties and extended plasma half-life in animal model systems, while maintaining potency towards wild-type and mutant viruses. We report here the design, synthesis and characterization of DMP 450, a water soluble cyclic urea with significant oral bioavailability in animal species ranging from rat to man. Plasma levels (which take into account plasma-protein-bound drug) in man after single doses of 10 mg kg^{-1} are maintained above the concentration required to inhibit not only laboratory strains and clinical isolates of HIV, but also mutant viruses with single amino acid changes at residues corresponding to the S1/S1' pocket of the protease.

To examine the relative potency of DMP 450 *in vivo*, we developed an assay system in which active protease expressed from recombinant plasmids injected into mouse skeletal muscle leads to loss of a heterologous plasmid-based reporter signal. DMP 450 is effective in this model system after intramuscular or oral dosing to the mice. Tissue culture studies in which HIV-infected cells are continuously exposed to DMP 450 indicate that multiply mutant variants of HIV can emerge, albeit slowly, and show considerable resistance to DMP 450. Three mutations appear to be required for significant (> 50-fold) attenuation of drug sensitivity. Taken together, these results suggest that cyclic ureas are a template that can provide clinically useful antiretroviral agents, and define an important and ambitious set of criteria for further optimization of cyclic urea inhibitors of the HIV protease.

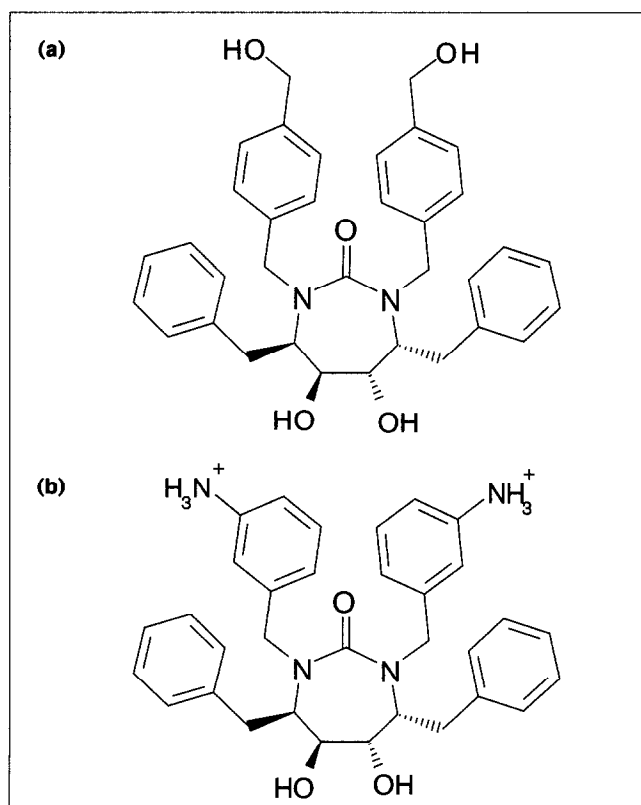
Results

Synthesis of DMP 450

We observed that symmetrical substitution of the urea nitrogens with *meta*- or *para*-substituted benzyl groups led to moderate increases in potency relative to small alkyl substituents (P.Y.S.L. *et al.*, unpublished data). If the benzylic substituent contained a hydrogen bond donor, as

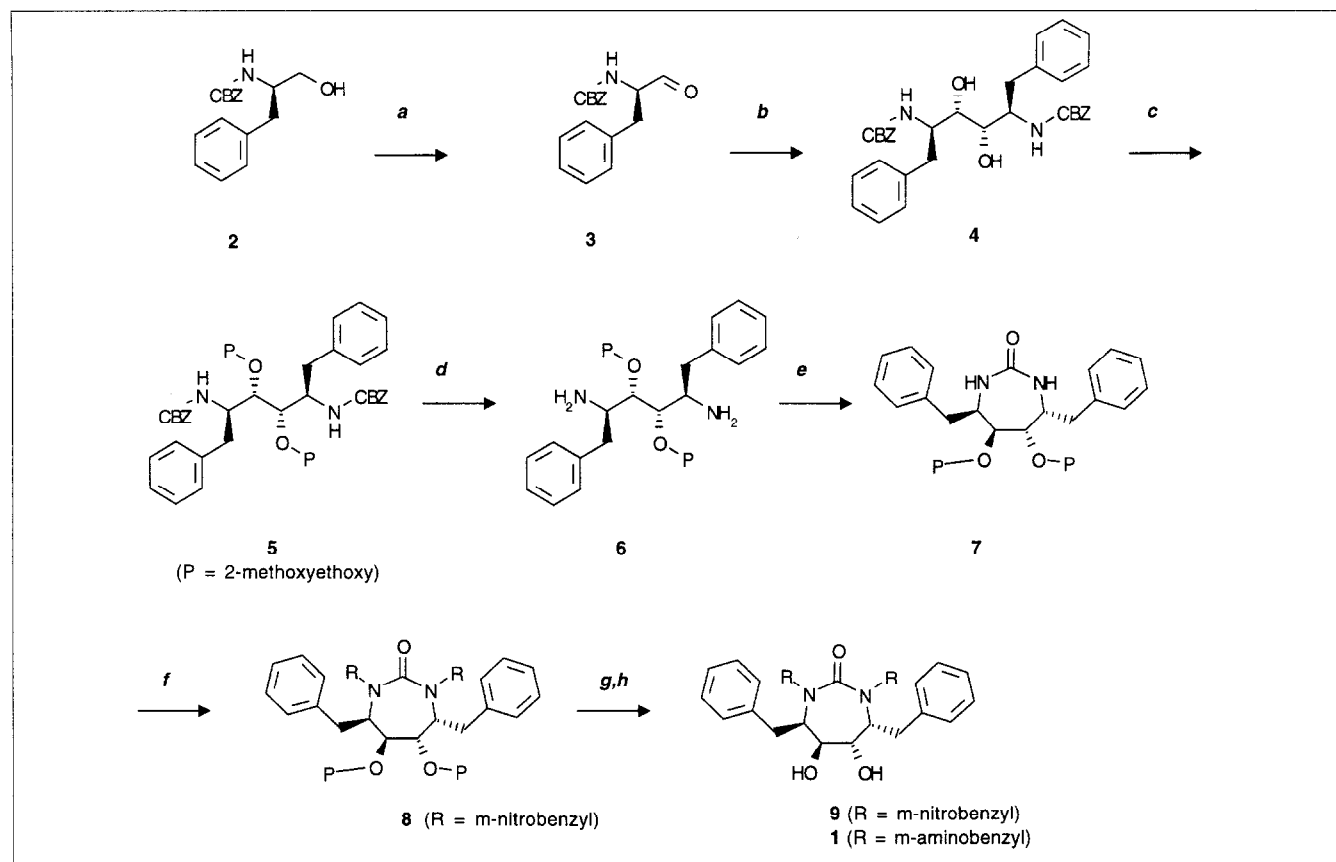
it does, for example, in DMP 323 (Fig. 1a), ~10-fold increases in both the enzymatic and antiviral potency resulted [23]. Since the unsatisfactory oral bioavailability in man of DMP 323 probably resulted from its poor solubility in both aqueous and lipid milieu, we sought to design benzylic-substituted cyclic ureas with acidic or basic functionalities. Basic groups enhance water solubility due to protonation. Attempts to introduce highly basic groups were uniformly unsuccessful, however, because in such structures a formal charge would need to have its associated water hydration shell stripped away before being forced into the solvent-sequestered S2/S2' pocket. For example, the *meta*-aminomethylbenzyl analog has more than 1000-fold weaker enzyme inhibitory activity than the isosteric *meta*-ethylbenzyl analog (data not shown). The weakly basic bis-*meta*-aminobenzyl derivative (DMP 450, compound 1, Fig. 1b), however, showed enhanced affinity relative to its tolyl counterpart, perhaps because it binds to the enzyme in the neutral form, but can form an ionic interaction with Asp30 and Asp30' (see below). The first pK_a of the anilino residue was expected to be high enough to be protonated in the stomach and for formulation as salts of strong acids, but low enough to be neutral in the cytosol, and this turned out to be the case (measured $\text{pK}_a = 4.6$).

Figure 1



Structures of (a) DMP 323 and (b) DMP 450.

Figure 2



Synthesis of DMP450. a: $(\text{COCl})_2$, DMSO, CH_2Cl_2 , Et_3N , -78°C , 84%. b: Zn^0 , $\text{CCl}_3(\text{THF})_3$, CH_2Cl_2 , room temperature (rt), 55% (pure RSSR isomer). c: DEA, MEM-Cl, reflux, 81%. d: 1 atm H_2 , 10% Pd/C,

THF, rt. e: THF, 1,1'-carbonyl-diimidazole, rt, 76%. f: NaH, m-nitrobenzyl bromide DMF, rt. g: MeOH, HCl, H_2O , rt. h: EtOH, 1N HCl, H_2 , Pd/C.

For synthesis of laboratory quantities of DMP 450, we used vanadium-mediated pinacol coupling [28] of N-carbobenzoxy-D-phenylalanyl-D-phenylglycerol-1,2-diol, as previously described [23,29–32] (Figure 2). The Materials and methods section provides full experimental procedures for the synthesis of cyclic ureas, some details of which have previously been published only in the patent literature [30,31]. The N-protected D-amino alcohol, required to set the stereochemistry of the ring substituents, was purchased in high optical purity (see Materials and methods). Oxidation, coupling, protection of the diol and deprotection of the amines, and cyclization with carbonyl diimidazole proceeded in acceptable yields. Alkylation with 3-nitrobenzyl bromide followed by reduction of the nitro groups and deprotection of the diol yielded pure DMP 450 in multigram quantities after chromatography or recrystallization. Modifications of this procedure for larger-scale preparation will be described elsewhere.

Solubility

The following amounts dissolved in 1 ml neutral water (range due to different recrystallization solvents): free base,

0.004–0.005 mg; bis-HCl salt, 18–45 mg; bis-mesylate salt, >130 to >170 mg.

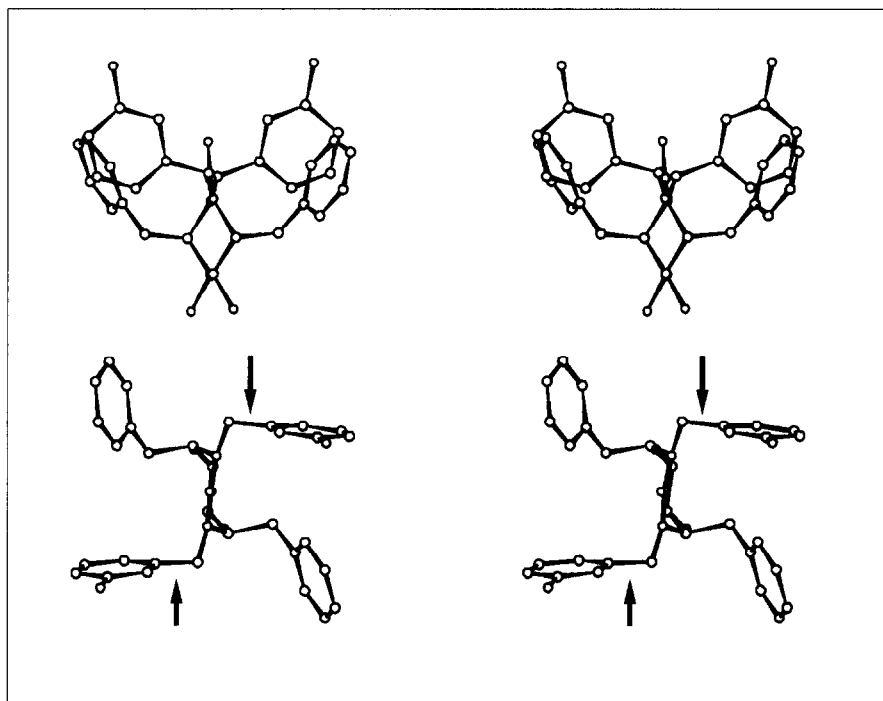
Crystal structure of DMP 450

Structural characterization of compounds 1, $1 \cdot (\text{MsOH})_2$ and $1 \cdot (\text{HCl})_2$ were carried out using standard X-ray crystallographic techniques (see Materials and methods). Two stereo views of the structure are shown in Figure 3. Normally, crystal packing contacts in cyclic ureas occur between the carbonyl and the hydroxyls (C.N.H. *et al.*, unpublished data); in DMP 450, the protonated anilines are turned upwards to hydrogen-bond to crystallographic water, which in turn H-bonds to the hydroxyls. The conformation shown is similar to the conformation in complex with HIV protease, except that the aniline rings are rotated almost 90° (see Fig. 3).

Inhibition of HIV protease by DMP 450 *in vitro*

DMP 450 is a potent inhibitor of HIV-1 protease as measured using peptide substrates or the HIV-1 Gag polyprotein as a substrate (Table 1). The differences in potency values obtained in the two assays reflect

Figure 3



Single crystal X-ray structure of DMP 450 (bis-hydrochloride salt). Two stereo views are shown, one with the O=C bond on the Z axis, and one with the CHOH-CHOH bond on the Z axis. The counterions and water in the unit cell are not shown. In the complex with HIV protease, the aniline rings are rotated almost 90° around the bond between the benzylic and aromatic carbon (arrows); otherwise the conformations are nearly identical (see later).

differences in buffer composition, pH and concentration of protease used. DMP 450 is a competitive inhibitor of HIV-1 protease, with rapid onset of inhibition (data not shown), as shown previously for the related cyclic urea DMP 323 [25]. As predicted for cyclic urea inhibitors, which incorporate a surrogate for the retrovirus-specific structural water molecule [23], DMP 450 inhibits mammalian aspartic proteases only weakly (17 % inhibition of pepsin at 1.2 mM DMP 450, 3.5 % inhibition of cathepsin D and 54 ± 33 % inhibition of renin, both at 120 μM), and failed to inhibit human pancreatic chymotrypsin and the chymotrypsin-like enzyme, neutrophil cathepsin G (0 % inhibition at 120 μM). The substrate specificities of the latter enzymes overlap that of HIV protease.

Inhibition of HIV replication

The measured concentrations required for 90 % inhibition (IC_{90} s) for four different types of antiviral assay and a variety of cell types and HIV isolates are shown in Table 1. DMP 450 is potent against laboratory strains of HIV-1 and HIV-2 as well as primary, clinical isolates derived from AZT-resistant patient samples. The concentration required to inhibit protease-mediated Gag polyprotein processing in persistently infected Molt-4 cells (0.24 μM) was similar to that required for antiviral activity as measured by reduction in the yield of infectious particles or in the level of viral RNA or p24 Gag protein. Thus the antiviral effect can be fully explained by inhibition of the viral protease in the infected cells. The

average value for IC_{90} against all HIV strains/cell types for all assays examined was 144 ± 52 nM. Cell viability assays demonstrated that the concentration required to reduce viability by 50 % (TC_{50}) for DMP 450 in MT-2 cells was greater than 80 μM . In addition, DMP 450 shows a large dynamic range of dose dependent inhibition of virus replication, reducing the yield of infectious virus by more than three orders of magnitude at concentrations as low as 0.5 μM (data not shown).

To estimate the influence of plasma-protein binding on the ability of DMP 450 to inhibit replication, the two major components of human plasma, human serum albumin and α -1-acid glycoprotein, were added to the tissue-culture medium, and the IC_{90} was measured for production of viral RNA or infectious virus. The IC_{90} for wild-type virus was 4.5-fold higher in the presence of these serum components when viral RNA was measured, and 8.4-fold higher in the presence of serum components when the yield of infectious progeny was measured by plaque assay. When lymphoid or monocytoid cell lines persistently infected with HIV-1 or HIV-2 were exposed to DMP 450 for four days at a concentration of 8.2 μM , progeny virion particles with morphology characteristic of immature particles, with uncondensed, partial cores, were observed (Fig. 4).

Resistance profile

Selected or recombinant viruses with amino acid substitutions corresponding to residues in the S1, S1' and S2, S2'

Table 1

Potency of DMP 450.			
Parameter		Mean \pm S.D.	
Potency against HIV protease			
K _i versus peptide substrate		0.28 \pm 0.09 nM	
IC ₅₀ versus Gag substrate		76 \pm 54 nM	
Virus	Cells	Assay	IC ₉₀ (μ M)
Potency against wild-type HIV			
HIV-1 (RF)	MT-2	Yield ^a	0.13 \pm 0.01
HIV-1 (RF)	MT-2	RNA ^b	0.15 \pm 0.08
HIV-1 (III B)	MT-2	Yield	0.16 \pm 0.08
HIV-1 (RF)	Molt-4	RIP ^c	0.24
HIV-1 (RF)	PBMC	Yield	0.10 \pm 0.04
HIV-1 (RF)	PBMC	p24 ^d	0.12
HIV-1 (RF)	U937	RIP	0.17
HIV-2 (ROD)	MT-2	Yield	0.18 \pm 0.06
AZT ^e -E ^e	MT-2	Yield	0.15 \pm 0.01
AZT ^e -E	PBMC	p24	0.04
Potency against mutant HIV			
sVal82Ala (RF/P9941)	MT-2	Yield	0.36 \pm 0.12 (2.8-fold incr.)
sVal82Phe/Leu97Val (virus E/DMP 323)	MT-2	Yield	1.28 \pm 0.25 (8.5-fold incr.)
sVal82Phe/Ile84Val (RF/DMP 323)	MT-2	Yield	15.0 \pm 0.94 (100-fold incr.)
rHxB2	MT-4	ICC	0.12 \pm 0.03
rVal82Phe/HxB2	MT-4	ICC	0.56 \pm 0.42 (4.7-fold incr.)
rIle84Val/HxB2	MT-4	ICC	0.88 \pm 0.41 (7-fold incr.)

^aThe yield of infectious particles was measured via plaque assay as described [26].

^bViral RNA in infected cultures was measured via oligonucleotide capture/hybridization [52].

^cViral Gag polyproteins were measured by radioimmunoprecipitation assay in chronically infected cells [27].

^dViral p24 antigen was measured by ELISA (DuPont NEN p24 Assay Kit).

^eHIV isolate from AZT resistant patient peripheral blood mononuclear cells, IC₉₀ for AZT > 4 μ M).

pockets of the HIV-protease dimer showed a lowered sensitivity to DMP 450, ranging from 2.8-fold (for a selected virus with a Val82Ala mutation) to 7-fold (for a recombinant virus with a Ile84Val mutation) increase in IC₉₀ for mutant variants with a single amino acid substitution. A 100-fold decrease in potency was, however, seen when recombinant virus selected *in vitro* against the related cyclic urea DMP 323 [33] was used. This *in vitro* selected virus carries mutations corresponding to changes in amino acids 82 and 84, which occupy the S1/S1' pockets and overlap with the S2/S2' pockets in the protease dimer.

To directly identify variants of HIV that may arise under selective pressure by DMP 450, two different isolates of HIV-1 were passaged in the presence of gradually

increasing concentrations of DMP 450, as described previously for the *in vitro* selection of DMP 323-resistant variants [33]. At selected passages, the sensitivity of virus to inhibition by DMP 450, and the sequence of the protease gene of the virus present, was determined (Table 2). Donor E, an AZT-resistant clinical isolate [33] that has a wild-type phenotype with respect to protease-inhibitor susceptibility, was passaged 29 times in increasing concentrations of DMP 450. Significant loss of sensitivity to DMP 450 (45-fold increase in IC₉₀) was associated with the presence of two to five mutations, including the Ile84Val mutation. Sequencing of virus with intermediate losses in sensitivity to DMP 450 inhibition suggests that mutation at position 46 is an early event, followed by mutation at position 84 and subsequently by mutation at positions 82, 90 and 45 (Table 2). HIV H9466, which is also wild-type with respect to protease-inhibitor susceptibility [34], was passaged 29 times with increasing concentrations of DMP 450. Significant loss in virus sensitivity to DMP 450 inhibition was associated with the presence of four mutations, including a mutation of Ile84Val.

Pharmacology profile

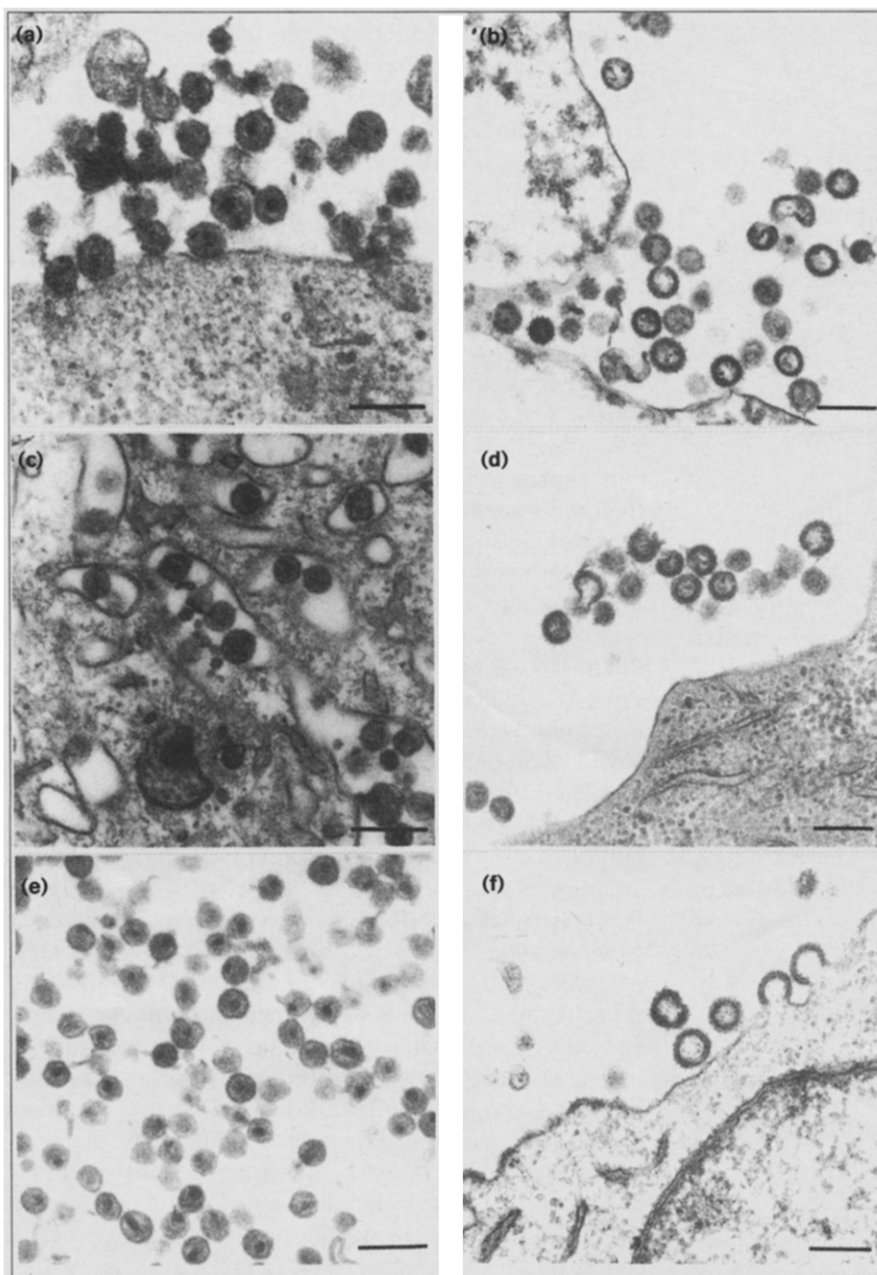
Prior to conducting phase I studies in HIV seronegative, male volunteers, the effects of single and chronic dosing of DMP 450 in rodents and dogs was determined. No significant adverse effects were noted in these preliminary pharmacological assessments. Complete details will be published elsewhere.

Pharmacokinetics of DMP 450 after oral and intravenous administration

After IV administration of 10 mg kg⁻¹ of DMP 450 to animals, plasma concentrations declined in a multiexponential fashion with terminal half-life values ($t_{1/2}$) ranging from 0.8 (rhesus monkeys) to 3.6 h (dogs) (Table 3). Apparent steady-state volume of distribution (V_{ss}) was larger in all species than that expected for distribution to the total body water space (0.7 l kg⁻¹) indicating extensive tissue distribution. After oral administration of a 10 (11 for human) mg kg⁻¹ dose, the time after dosing when highest plasma concentrations were achieved (t_{max}) varied among species ranging from 0.5 (rats) to 2.0 h (dogs) (Table 3). Under ketamine sedation in the chimpanzee (10 mg kg⁻¹), t_{max} was delayed to 8 h postdose. The highest plasma concentration achieved after dosing (C_{max}) was greatest (11.19 μ M) in dogs, and lowest (1.53 μ M) in chimpanzees (Table 3). Bioavailability (the fraction of the dose absorbed into the bloodstream (F%)) ranged from 23.8 % in chimpanzee to 79.3 % in the dog (Table 2). Plasma concentration versus time profiles for comparable perioral (PO) doses in all species are shown in Figure 5.

Effect of oral DMP 450 administration on HIV protease *in vivo*

Direct injection of expression plasmids for luciferase and HIV protease into mouse quadriceps muscle resulted in

Figure 4

Treatment of HIV-infected cells with DMP 450 causes production of immature virus particles. Electron micrographs of various cell lines chronically infected with HIV and treated for 4 days with DMP 450 are shown. Panels (a) and (b), Molt-4 cells infected with HIV-1(RF); Panels (c) and (d), U937 cells infected with HIV-1(RF); Panels (e) and (f), H9 cells infected with HIV-2(ROD). (a),(c),(e), untreated controls; (b),(d),(f), cells treated with 8.2 μ M DMP 450. Bar = 200 nm.

significantly decreased luciferase activity in muscle extracts compared to injection of luciferase plasmid alone (Table 4). We did not see this effect when the protease expression plasmid encoded HIV-protease containing a single, inactivating amino acid substitution at the active site (Asp25Gly), indicating that the reduction in luciferase signal was due to HIV protease co-expression. When injected directly into the quadriceps muscle (10 μ g) or when administered orally at 100 mg kg⁻¹, DMP 450 provided complete protection of the luciferase signal. In contrast, DMP 323 caused some recovery of luciferase activity when injected intramuscularly,

but failed to protect the luciferase signal at all when administered twice daily (BID), even at 100 mg kg⁻¹ or PO at 200 mg kg⁻¹ (B.K., *et al.*, unpublished data).

X-ray crystal structure of HIV-protease-DMP 450 complexes

As shown in Figure 6, the bound DMP 450 links the active site aspartates to the flexible loops via a hydrogen bonding network without an intervening water molecule. The amine nitrogen of the m-aminobenzyl substituent is located within hydrogen bonding distance of the side chain of Asp30 and Asp30' of the protease dimer. The benzyl groups

Table 2***In vitro* selection of DMP 450-resistant variants.**

Passage	IC ₉₀ ($\mu\text{g ml}^{-1}$)	Fold increase in IC ₉₀	Amino acid sequence
Donor E (initial)	0.065	1.0	Wild type
Donor E Passage 18	0.665	10.2	Met46Leu Val82Val/Ile (mixture) Ile84Ile/Val (mixture)
Donor E Passage 29	2.95	45.4	Lys45Lys/Ile (mixture) Met46Leu Val82Val/Ile (mixture) Ile84Val Leu90Leu/Met (mixture)
H9466 (initial)	0.095	1.0	Wild type
H9466 Passage 12	0.917	9.7	Asp60Glu Ile84Val
H9466 Passage 29	5.67	59.7	Leu10Phe Met46Ile Asp60Glu Ile84Val

vicinal to the diol oxygens occupy the S1/S1' pockets as observed previously [23,35,36]. DMP 450 thus binds symmetrically to the HIV-protease dimer, in contrast to the asymmetric interactions reported for most [37–39], but not all [40] linear diol inhibitors of the enzyme. In addition to the steric fit of DMP 450 into the active site, the four strong hydrogen-bonding interactions (carbonyl, diol, anilines) provide a remarkable electrostatic complementarity that anchors and perhaps guides the inhibitor into place (Fig. 7).

Discussion

The recent studies of Ho *et al.* [41] and Shaw and colleagues [42] and their analysis by Coffin [43] have

demonstrated that the HIV-seropositive individual is in a state of metastable equilibrium between HIV-mediated T-cell destruction linked to high levels of virus replication, and T-cell regeneration. An effective therapy must shift this balance in favor of cell repletion through significant reductions in the viral burden without concomitant enrichment and/or *de novo* generation of resistant mutant HIV if therapeutic benefit is to be sustained. Recent clinical trial results with the potent protease inhibitors Saquinavir [12,44], Indinavir [10,42], and Ritonavir [11,21,41,42], in which antiviral efficacy diminishes with treatment time, have shown that oral bioavailability and the ability to block the generation and/or outgrowth of resistant mutants of HIV are important in the clinical outcome of therapy as measured by plasma viral burden. The combined use of protease inhibitors and reverse transcriptase inhibitors is currently the most potent clinical option for relief of viral burden; long term efficacy and safety of such combinations is under investigation. We have focused on oral bioavailability and characterization of resistance profiles without sacrifice of sub-micromolar potency as key criteria in identifying a suitable clinical candidate within the cyclic urea series of protease inhibitors. This early focus on bioavailability together with the synthetic flexibility for introducing P2 and P2' substitutions onto the rigid, cyclic urea core structure have resulted in the design of DMP 450, a small, water soluble, HIV-protease inhibitor that is highly orally bioavailable.

At sub-micromolar concentrations, DMP 450 is a potent inhibitor of the replications of laboratory strains and nucleoside-sensitive and resistant clinical isolates of HIV. The suppression of virus replication — measured by quantitation of viral RNA, p24 antigen, or production of infectious particles as assessed by plaque assay — correlated with the ability of DMP 450 to block processing

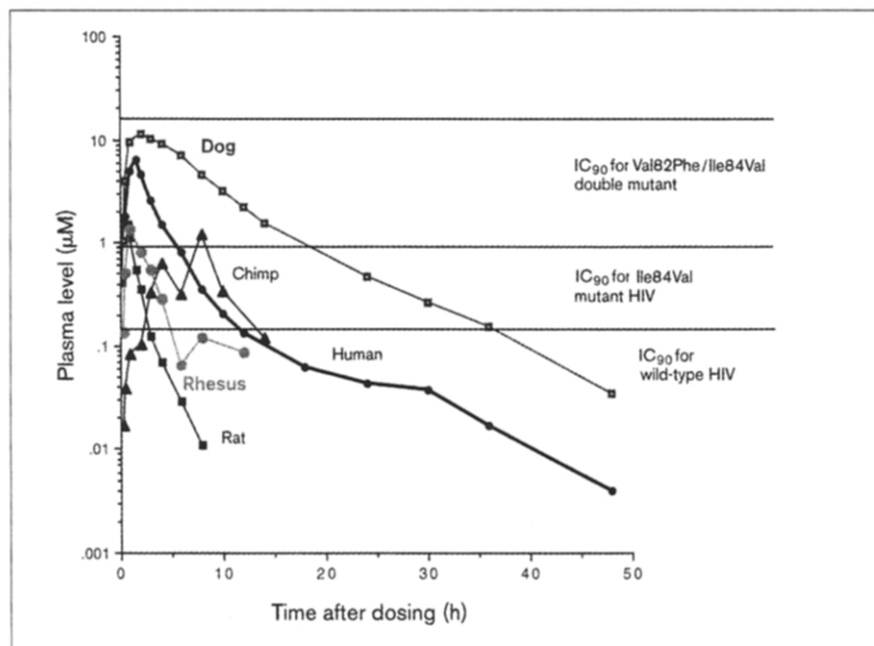
Table 3**Pharmacokinetics of DMP 450.**

Parameter	Rat	Dog	Rhesus monkey	Chimpanzee ^a	Human
IV dose (mg kg ⁻¹)	10	10	10	10	—
Cl (l h ⁻¹ kg ⁻¹)	4.7 ± 0.77	0.21 ± 0.06	1.20 ± 0.35	0.72 ± 0.15	—
V _{ss} (l kg ⁻¹)	4.52 ± 0.42	1.35 ± 0.48	1.37 ± 0.32	1.57 ± 0.39	—
t _{1/2} (h)	1.3 ± 0.2	3.6 ± 1.0	0.8 ± 0.1	2.6 ± 1.0	—
PO dose (mg kg ⁻¹)	10	10	10	10	11 ^b
C _{max} (μM)	2.25 ± 0.72	11.19 ± 1.33	1.60 ± 1.67	1.53 ± 0.92	6.49 ± 1.89
t _{max} (h) (range)	0.5 (0.25–0.75)	2.0 (1.0–2.0)	1.5 (1.0–3.0)	8.0 (4.0–8.0)	1.5 (1.0–1.5)
F %	70.5 ± 21.8	79.3 ± 13.6	25.7 ± 17.4	23.8 ± 5.3	ND

^aChimpanzees were lightly sedated with 10 mg kg⁻¹ ketamine prior to dosing and before each specimen collection.

^bAll subjects were given a fixed dose of 750 mg, average body weight was 67.9 kg.

Figure 5



DMP 450 displays oral bioavailability in several species. The pharmacokinetics of DMP 450 after oral dosing is shown. Groups of animals ($n = 4-8$) or healthy, HIV-seronegative human subjects ($n = 8$), were given DMP 450 in solution or as neat powder in capsules (see text). The dose was 10 mg kg^{-1} for all species except human, where the dose was 11 mg kg^{-1} . Plasma concentrations were determined by HPLC, and are expressed as μM .

of the Gag polyprotein produced in chronically infected T-cells or macrophages and with the formation of immature particles with the characteristic non-condensed internal structure [45]. Previous studies with the related cyclic urea DMP 323 demonstrated that extended incubation of such immature particles after removal of the compound to a level below that required for significant inhibition of replication *in vitro* did not result in

maturation of the particles [27], suggesting that the timing of assembly events is critical for the formation of infectious, mature particles. Similar experiments with DMP 450 indicate that this cyclic urea also blocks polyprotein maturation such that maturation of latent particles does not occur after removal of the compound.

Table 4

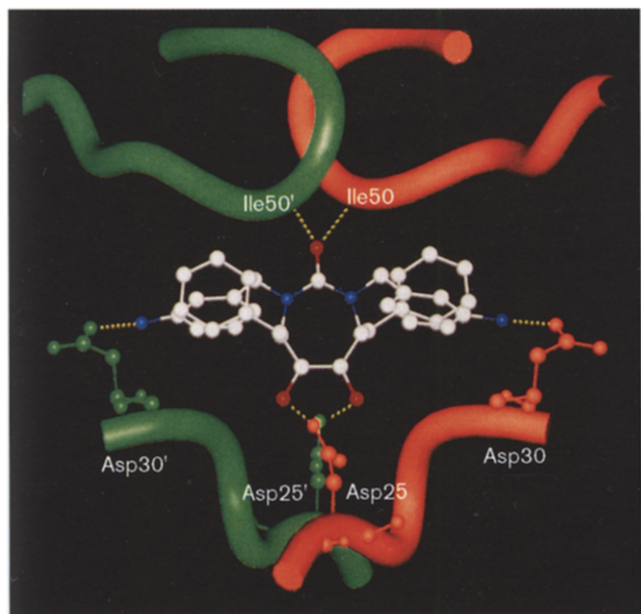
Activity of firefly luciferase in mouse muscle extracts.

Plasmid(s) injected	Luciferase activity ^a (light units mg^{-1})
No plasmid	<100
LUC plasmid alone	79 208
LUC + HIV protease	9 504
LUC + HIV protease Asp25Gly	73 712
LUC + HIV protease + DMP 323 (IM, $10 \mu\text{g}$)	76 782
LUC + HIV protease + DMP 450 (IM, $10 \mu\text{g}$)	74 183
LUC + HIV protease + DMP 323 (PO, 100 mg kg^{-1})	11 530
LUC + HIV protease + DMP 450 (PO, 100 mg kg^{-1})	74 863

^aThe quadriceps muscles of Balb C mice were injected with the indicated plasmids either with or without concomitant dosing by the indicated route with cyclic ureas DMP 323 or DMP 450 as described in Materials and methods. After 48 h, luciferase activity was measured in quadriceps homogenates using firefly luciferin as substrate in the presence of ATP. Activity is expressed as light units per mg of muscle extract.

DMP 450, like its predecessor DMP 323, showed a high degree of specificity for the HIV protease in comparison with other mammalian aspartic proteases and chymotrypsin-like enzymes. This exquisite selectivity for HIV protease was predicted, since the cyclic urea framework incorporates the hydrogen-bonding equivalents of a structural water molecule that is only present in retroviral proteases [1-4].

DMP 450 seems to have a resistance 'profile' qualitatively similar to that of DMP 323, consistent with the similarity of the X-ray structures of co-crystals of DMP 323 or DMP 450 with the HIV-protease dimer. The aromatic rings occupying the S1/S1' pockets form hydrophobic interactions with the sidechains of Val82 and Ile84 that would be disrupted with mutations of these residues to the bulkier Phe or smaller Ala at position 82 or to the smaller Val at position 84. In contrast to DMP 323, where the *para*-hydroxymethylbenzyl groups on the urea nitrogens form hydrogen bonds with backbone amides between Asp29 and Asp30 and Asp29' and Asp30', the *meta*-oriented amino group of DMP 450 allows for a (presumably) ionic hydrogen bond to the side chains of Asp29 and Asp29'. For the recombinant and selected

Figure 6

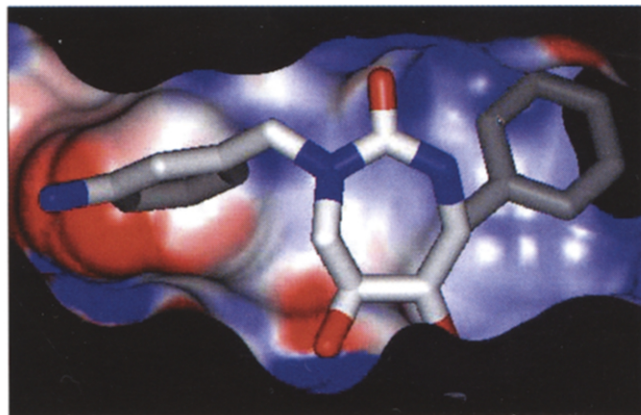
DMP 450 binds symmetrically to the HIV-protease dimer. The X-ray crystal structure of DMP 450 bound to the active site of the HIV protease dimer is shown. All heavy atoms of the inhibitor are shown in ball and stick representation, and a selected portion of the protein backbone is shown as a ribbon; red for monomer 1 and green for monomer 2. The side chains of the active site aspartates, Asp 25 and Asp 25', and of Asp 30 and Asp 30' are also depicted in ball and stick representation. The urea oxygen of DMP 450 forms two long hydrogen bonds with flap residues Ile50 and Ile50'. The nitrogens of DMP 450 are shown in blue, oxygens in red. Hydrogen bonds are depicted by dashed lines.

viruses examined, single amino acid substitutions at position 82 or 84 result in a two- to seven-fold loss of sensitivity to inhibitor. These mutants can arise from wild type HIV by a single base pair change, and are thus likely to be present within the quasi species mixture that is now believed to characterize the HIV infected individual [43].

The double mutant Val82Phe/Ile84Val, which was identified in tissue culture following prolonged growth of HIV in the presence of DMP 323, has a greater reduction in sensitivity to DMP 450 than the product of the resistance observed for the single mutations alone. Efforts to understand the structural basis for this large loss of apparent binding affinity are currently in progress. These two mutations do not appear to account for all of the changes in the DMP 323-selected virus, however. When the corresponding mutations were introduced into the HxB2 background, we could not recover infectious virus particles in the usual time required for mutant virus outgrowth, despite confirming proper plasmid construction and lipofection efficiency. This suggests that other mutational events, including changes outside the protease coding region, may have

altered the relative replication competency of the double mutant during the selection experiments, such that it survives to become the majority population during the extended selection process. Such mutations might give rise to subtle conformational effects or encode outright changes in the processing-site sequence in the polyprotein substrate. Lamarre *et al.* [46] have recently presented evidence supporting the possibility that mutation of protease-processing sites is linked with the development of resistance-conferring mutations within the protease gene itself.

A significant property of DMP 450 which is directly relevant to its potential for clinical efficacy is the substantial oral bioavailability observed in all species examined, including man. In single-dose pharmacokinetic studies, when delivered as a clear aqueous solution (pH ~3) or as neat powder in gelatin capsules, plasma levels remained above the IC_{90} for wild-type HIV in all species for at least 3 h, and more than 10 h in dog, chimp, and human. Plasma levels remained above $1.3 \mu\text{M}$, which is the highest IC_{90} for known mutant HIVs containing single substitutions at position 82 or 84, for 4 h or more in

Figure 7

DMP 450 is electrostatically complementary to the HIV protease active site. The coordinates from the X-ray crystal structure were used to generate this graphical representation: hydrogens were added at pH 6.0 and partial and formal charges were assigned using InsightII (Biosym Technologies Inc., San Diego, CA). The catalytic aspartic acids were assigned a zero formal charge as determined by NMR spectroscopy of the DMP 323-HIV protease complex [35]. The electrostatic potential of the HIV protease in this model, with no inhibitor in the active site, was calculated using Delphi [59] and a water-accessible surface area of the binding site within 4 Å of DMP 450 was color-coded by an electrostatic potential spectrum (blue = $2 \text{ kcal mol}^{-1} \text{ e}^{-1}$; white = 0; red = $-2 \text{ kcal mol}^{-1} \text{ e}^{-1}$). The inhibitor is color-coded by atom type to show the interaction of the oxygens and nitrogens with complementary charged regions of the enzyme active site. The P1 and P2' benzylic groups are omitted for clarity. Note that the hydrogen atoms of the diol are not shown; these form an extensive hydrogen-bonded array with Asp25/25' and Gly27/27', and mediate the interaction between the diol oxygens and the proximal, negatively charged (red) regions of the catalytic triad.

human and dog at these single doses. When we adjust the IC₉₀ values shown in Figure 5 for the 4–8-fold increase measured in the presence of human serum components, the plasma levels after acute dosing exceed even the adjusted IC₉₀ for the Ile84Val mutant virus in the dog and in man for at least 2 h.

In the clinical setting, it is the concentration of intracellular free, or 'available', compound concentration that will determine the level of inhibition observed and the resulting level of efficacy. Indeed, excessive plasma protein binding can lead to clinical failure, as recently observed with the HIV protease inhibitor SC-52151 [47]. DMP 450 binds to human plasma proteins, as determined by equilibrium dialysis using undiluted human plasma and 5 µg ml⁻¹ of ¹⁴C-labeled compound, to the extent of 90–93 %. This extent of binding is consistent with the 4–8-fold upward-shift in IC₉₀ measured in antiviral assays. The quantitative effect that this degree of protein binding, determined in a static system, will have on availability of compound for entry into cells and inhibition of protease within the HIV-infected individual remains to be determined. It was thus of interest to determine the potential for HIV-protease inhibition in an *in vivo* system used as a model of the complex and multi-compartment milieu encountered by an orally dosed pharmaceutical.

Previous studies by Wolff *et al.* [48,49] have established that manual injection of plasmid DNA, encoding reporter genes, directly into striated muscles causes efficient synthesis of the reporter protein. Preliminary experiments in mammalian cell lines indicated that transient co-expression of the HIV-1 protease with a reporter protein such as firefly luciferase caused large reductions in the quantity of reporter activity as a result of protease activity in the cells; this effect could be blocked by treating the cells with protease inhibitors (B.K. *et al.*, unpublished data). Based on the results of these transfection experiments, we have used the gene transfection technology of Wolff *et al.* to co-express HIV protease and luciferase in mouse muscle following injection of the corresponding plasmids. We then assessed the effect of orally or intramuscularly administered DMP 450. We also compared the relative potency of DMP 450 and DMP 323, which has a similar protein binding profile (88 %, [24]) but significantly lower bioavailability. Complete protection against protease-mediated loss of reporter signal was observed with local (intramuscular) injection of either cyclic urea, but only with DMP 450 when the compounds were administered orally.

Orally-delivered DMP 450 but not DMP 323 also delays HIV-protease mediated cataract development in a transgenic mouse line in which the HIV-protease dimer is expressed from a transgene controlled by the lens α-crystalline promoter (Jonak, G.J., *et al.*, (1993). EMBL

Meeting on Mouse Molecular Genetics, Aug. 18–22, 1993, Abstract 258). Preliminary pharmacokinetic experiments in mice indicate that peak plasma levels of 7.2 µM and 4 h levels of 0.18 µM (total drug concentrations) are achieved following a 100 mg kg⁻¹ PO dose of DMP 450. We have used a radioimmunoprecipitation (RIP) assay on extracts of cells in culture to determine the efficacy of the cyclic urea compounds in culture medium against the protease in an intracellular environment (Table 1 and [27]). If one assumes that the IC₉₀ value obtained in these studies defines the plasma level of available drug that would be required to inhibit HIV replication *in vivo*, a level of ~0.2 µM would be required for either cyclic urea to yield 90 % inhibition. Our animal experiments indicate that measurable inhibition of protease activity within cells occurred with dosing regimens that resulted in total blood levels that exceeded the IC₉₀, determined by RIP *in vitro*, for at least part of the dosing interval.

Taken together, the potency towards wild-type and mutant HIV, the pharmacokinetic profiles we have observed for single doses of DMP 450, and the desirable physicochemical properties for an oral, solid-dosage form suggested the potential for clinical efficacy for this cyclic urea inhibitor of the HIV protease. In clinical trials to date with HIV-protease inhibitors, however, the initial efficacy as documented by viral load measurements is transient under several dosing regimes, due to the outgrowth of resistant virus under the selective pressure of drug therapy [21,41,42]. Whereas DMP 450 is effective against diverse wild-type isolates and against some mutants carrying single amino acid mutations within the protease, sensitivity of virus towards inhibition by DMP 450 was decreased in mutants harboring the Ile84Val mutation and was substantially lowered in the presence of two or more mutations. Although viruses selected *in vitro* after exposure to the two related cyclic ureas DMP 323 [32] or DMP 450 (Table 2) were not identical, the Ile84Val mutation was present in all of them.

Rose *et al.* [50] recently examined the influence of virus background on the vitality of mutant HIV variants; they propose that some mutant variants assembled and/or analyzed *in vitro* may never arise *in vivo*, depending on the background amino-acid sequences surrounding the mutations. This finding suggests that *in vitro* measurements of inhibitor potency towards mutant HIVs should be viewed with caution. But the fact that the Ile84Val mutation detected here was associated with loss of potency of DMP 450 in a variety of backgrounds, and was further aggravated by additional mutations, led us to anticipate that DMP 450 treatment would eventually lead to outgrowth of Ile84Val-containing viruses. Mutant viruses with changes at position 84 are also observed *in vitro* and *in vivo* in association with exposure to the HIV-protease inhibitors Ritonavir and Indinavir [16,17]. Although DMP 450

is continuing in clinical trials with another sponsor, we are now focusing on identifying a third-generation cyclic urea inhibitor of the viral protease that will achieve blood levels of free drug sufficient to suppress replication HIV variants containing one or more mutations in addition to the Ile84Val substitution. Such an inhibitor could in principle be used as a single therapy or combined with any of the current clinical candidates or with inhibitors of other retroviral enzymes.

Significance

This study highlights the successful application of structural, chemical and biochemical data to the discovery of an improved HIV protease inhibitor, and continues the iterative process of identifying the properties necessary for an effective AIDS therapy. The introduction of water-solubilizing ionizable groups onto the already compact and pre-organized cyclic urea HIV-protease inhibitor resulted in a potent and selective antiviral agent with extremely favorable pharmacokinetic properties. Preclinical and clinical testing of DMP 450 and other protease inhibitors has established the importance of maintaining enzyme inhibition across a broad spectrum of potential and actual HIV protease mutant variants. Resistant strains have been identified that are associated with significant losses in drug potency for all of the antiviral agents currently in clinical trials; it is therefore uncertain whether these drugs can provide a sustained decrease in viral load that will lead to significantly improved immunocompetency. The DMP 450 structure, and cyclic ureas in general, provide a rigid, well-characterized physicochemical framework to continue the search for the optimal compound for use as a long-term treatment of AIDS.

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Materials and methods

Preparation of DMP 450

All procedures were carried out under inert gas in oven-dried glassware unless otherwise indicated. ¹H-NMR spectra were obtained on VXR or Unity, 300 or 400 MHz instruments (Varian Instruments, Palo Alto) with tetramethylsilane as an internal reference standard. Melting points were determined on a Mettler SP61 apparatus and are uncorrected. Elemental analyses were performed by Quantitative Technologies, Inc., Bound Brook, NJ. High resolution mass spectra were carried out on a VG 70-VSE instrument with NH₃ chemical ionization. Thin-layer and column chromatography were carried out on plates or silica gel from E. Merck, Darmstadt, Germany. Separation of optical isomers was performed using supercritical fluid chromatography with a Chiracel OD (Daicel Chemical Industries Ltd) and 20 % methanol-modified CO₂ mobile phase. Solvents and reagents were obtained from commercial vendors in the appropriate grade and used without further purification unless otherwise indicated. (R)-(phenylmethyl)-[2-hydroxy-1-(phenylmethyl)ethyl]carbamate

(compound **2**) was purchased from Synthetech, Inc., Albany, OR; $[\alpha]_D^{25} = +40.9 \pm 0.8$ (C = 1.01 g per 100 ml EtOH).

Synthesis of (R)-(phenylmethyl) (1-formyl-2-phenylethyl)-carbamate (compound **3**)

A solution of 52 ml of oxalyl chloride (0.59 mol) in 500 ml dichloromethane (CH₂Cl₂) was cooled to -78 °C, and 57 ml anhydrous dimethylsulfoxide (0.81 mol) was added in 500 ml CH₂Cl₂ over 1 h while the temperature was maintained below -70 °C (caution: exothermic). Stirring was continued at -78 °C for 30 min and the alcohol **2** (125 g, 0.44 mol) was added in 800 ml CH₂Cl₂ over 1 h, again maintaining the temperature below -70 °C, followed by stirring for 30 min at -78 °C. Triethylamine (244 ml) was added in 300 ml CH₂Cl₂ over 0.5 h, followed by stirring for 2 h at -70 °C. After 800 ml of 20 % aqueous KHSO₄ was added, the reaction was allowed to warm to room temperature and 300 ml water was added. The aqueous phase was separated and washed with 400 ml CH₂Cl₂, and the combined organic layers from two such runs were washed with 1 l of saturated aqueous NaHCO₃, 1 l water and 1 l saturated aqueous NaCl, dried over MgSO₄ and concentrated to 700 ml. Hexane (2 l) was added, the mixture cooled in an ice bath for 1 h, and the solids were filtered and washed with cold hexane. After drying to constant weight at 40–50 °C, 209 g (84 % yield) of product was obtained, with a melting point (m.p.) of 82–83 °C; $[\alpha]_D^{25} = +53.7 \pm 0.8$ (C = 1.04 g per 100 ml EtOH); NMR (300 MHz, CDCl₃) 3.15 (d, J = 6 Hz, 2H); 4.5 (d, J = 12.6 Hz, 1H); 5.1 (s, 2H); 5.35 (m, 1H); 7.1–7.4 (m, 10H), 9.6 (br s, 1H). The aldehyde, when pure, could be stored under inert gas without racemization or trimerization. Runs were pooled as necessary for the following step.

Synthesis of [1R-(1R*,2S*,3S*,4R*)]-bis(phenylmethyl) 2,3-dihydroxy-1,4-bis(phenylmethyl)-1,4-butanediylbis[carbamate] (compound **4**)

A four-necked reaction flask was charged with VCl₃(THF)₃ (467 g, 1.25 mol) and 1 l CH₂Cl₂ in a dry box, removed from the dry box and fitted with a reflux condenser, a nitrogen bubbler, and a thermocouple. Zinc dust (Aldrich, 54 g, 0.83 mol, also weighed in the dry box) was added; the temperature rose to 40 °C. Aldehyde **3** (350 g, 1.2 mol) was added rapidly in 700 ml CH₂Cl₂, the temperature rose to 40 °C, and the reaction was stirred overnight. A flask containing 6 l water and 500 ml concentrated HCl was warmed until the solution reached about 65 °C. The reaction mixture was added to the aqueous acid by addition funnel, and CH₂Cl₂ was collected in a cooled flask as it distilled. When addition was complete, the remaining material was allowed to cool to room temperature and the precipitate was collected and washed with water until colorless, then with 1.2 l ethanol, followed by 1.2 l hexane. The crude product was dried on the filter at 75 °C. Two of the above runs were combined and recrystallized by dissolving in hot tetrahydrofuran (THF), filtering, adding hexane and allowing to cool. A total of 374 g (55 %) of a white crystalline solid was recovered, m.p. 211–213 °C. HPLC analysis showed 97 % (1R, 2S, 3S, 4R) isomer, 3 % (1R, 2R, 3R, 4R) isomer. HRMS: calc'd, 569.2651; found, 569.2644. Recrystallization (CHCl₃/hexane) gave analytically pure material: a white solid with m.p. 217–218 °C. Anal. calc'd. for C₃₄H₃₆N₂O₆: C, 71.81; H, 6.38; N, 4.93; found: C, 71.70; H, 6.43; N, 4.90.

Synthesis of [1R-(1R*,2S*,3S*,4R*)]-bis(phenylmethyl) 2,3-bis[(2-methoxyethoxy)-methoxy]-1,4-bis(phenylmethyl)-1,4-butanediylbis[carbamate] (compound **5**)

N,N-diisopropylethylamine (Aldrich; 1135 ml, 840 g, 6.5 mol) was added in one portion to a slurry of 600 g compound **4** (1.1 mol) in 5 l CH₂Cl₂ at room temperature followed by dropwise addition of 600 g 2-methoxyethoxymethyl chloride (MEM-Cl, TCI America, Portland, OR; 4.8 mol) over 1 h (caution: exothermic). The solution was heated at reflux for 12 h, followed by addition of 3 l ice water. The organic layer was separated and washed twice with 1.5 l water, then dried over magnesium sulfate. Solvent was removed to yield 890 g oil; this was

recrystallized with 1-chlorobutane/hexane to yield 660 g (81 %) of a white solid, m.p. 52–54 °C. ¹H NMR (300 MHz, CDCl₃): 2.8 (m, 4H, CH₂Ph); 3.38 (s, 6H, OCH₃); 3.58 (m, 8H, OCH₂CH₂O); 3.8 (m, 2H, CHOH); 4.2 (m, 2H, CHNH); 4.6–5.2 (m, 10H, NH, PhCH₂O, OCH₂O); 7.25 (m, 2H, C₆H₅). MS (ESI): 745 (M+1, 23 %). HRMS: calc'd, 745.3700; found, 745.3715. Re-recrystallization gave a white solid, m.p. 57–58 °C. Anal. calc'd for: C₄₂H₅₂N₂O₁₀: C, 67.72; H, 7.04; N, 3.76; found: C, 67.57; H, 7.06; N, 3.73

Synthesis of [2R-(2R,3S,4S,5R)]-3,4-bis[(2-methoxyethoxy)methoxy]-1,6-diphenyl-2,5-hexanediamine (compound 6) and [4R-(4a,5a,6b,7b)]-hexahydro-5,6-bis[(2-methoxyethoxy)methoxy]-4,7-bis(phenylmethyl)-2H-1,3-diazepin-2-one (compound 7)

THF (200 ml) and 20 g (26.8 mmol) of compound 5 were stirred under 1 atmosphere of hydrogen in the presence of 10 % palladium on carbon (Aldrich) for 7 h or until thin-layer chromatography (TLC; 10:1:10 ethyl acetate:ethanol:hexane, R_f = 0.05 for product) indicated that the reaction was complete. The suspension was filtered through a pad of diatomaceous earth and washed with 150 ml THF. CDI (1,1'-carbonyldiimidazole, Aldrich; 5.5 g, 33.3 mmol) was added directly to the solution in several portions as a solid followed by stirring at room temperature for 12 h or until the reaction was complete, as determined by TLC using the above solvent system (R_{f(product)} = 0.26). The mixture was quenched with 150 ml ice-cold 0.5N HCl and extracted twice with 50 ml diethyl ether; the combined organic phases were washed twice with 100 ml water and dried over magnesium sulfate. Removal of solvent and silica-gel chromatography (1:1 hexane:ethyl acetate followed by 10:1:10 acetate:ethanol:hexane) yielded 10.2 g (76 % yield from compound 5) of a colorless oil. ¹H NMR (CDCl₃): 2.90 (m, 4H, CH₂Ph); 3.36 (s, 6H, OCH₃); 3.4 (m, 8H, OCH₂CH₂O); 3.90 (t, 2H, OH); 4.10 (s, 2H, NH); 4.80 (q, 4H, OCH₂CH₂O); 7.3 (m, 10H, C₆H₅).

Synthesis of [4R-(4a,5a,6b,7b)]-hexahydro-5,6-bis(hydroxy)-1,3-bis[(3-nitrophenyl)methyl]-4,7-bis(phenylmethyl)-2H-1,3-diazepin-2-one (compound 9)

To a solution of 311 mg of MEM-protected cyclic urea 7 in 4 ml of dry dimethylformamide was added 248 mg of 60 % sodium hydride in mineral oil. The mixture was stirred for 15 min, and 804 mg of 3-nitrobenzyl bromide was added. The mixture was stirred for 16 h, hydrolyzed with water and extracted with ether to give an oil that could be purified by preparative TLC on silica gel with 60:40 hexane:ethyl acetate. A major fraction (R_f 0.14, 0.28 g, oil) was identified as [4R-(4a,5a,6b,7b)]-hexahydro-5,6-bis(2-methoxyethoxymethoxy)-1,3-bis[(3-nitrophenyl)methyl]-2H-1,3-diazepin-2-one by MS: (M + H)⁺ = 773.3400; calc'd., 773.3398. For preparative purposes the protected bis-nitro compound was not isolated, but the 2-methoxyethoxymethyl protective groups were removed directly by dissolving 173 mg of the above crude oil in 5 ml of 4N HCl/dioxane. After 16 h the mixture was evaporated, and the crude product was purified by preparative TLC on silica gel with 95:5 dichloromethane:methanol to give 0.11 g (R_f 0.56) of crystalline compound 9, m.p. 248.3 °C after recrystallization from ethanol. MS: (m + H)⁺ = 597.2349; calc'd., 597.2349.

Synthesis of [4R-(4a,5a,6b,7b)]-hexahydro-5,6-bis(hydroxy)-1,3-bis[(3-aminophenyl)methyl]-4,7-bis(phenylmethyl)-2H-1,3-diazepin-2-one (compound 1): free base, bis-hydrochloride, and bis-methanesulfonic acid salt (DMP 450)

A mixture of 110 mg of compound 9, 20 ml ethanol, 2 ml 1N HCl and 40 mg of 10 % Pd/C was hydrogenated at atmospheric pressure for 16 h. The product was purified by preparative TLC on silica gel with 90:10 dichloromethane:methanol to give 90 mg of the free base (R_f 0.41), m.p. 245.7 °C. MS: (m + H)⁺ = 537.2854; calc'd., 537.2866. Bis-hydrochloride salt, m.p. 200 °C (dec), [α]_D²⁵ = +107.28 (ethanol). Anal. calc'd. for C₃₃H₃₆N₄O₃·2HCl: C, 65.02; H, 6.28; N, 9.19, Cl,

11.63.; found: C, 65.03; H, 6.48; N, 9.07. Cl, 11.36. Bis-methanesulfonate salt (DMP450), m.p. 208.3 °C (dec). Anal. calc'd. for C₃₃H₃₆N₄O₃·2CH₄O₃S·3H₂O: C, 53.69; H, 6.44; N, 7.16; O, 24.52; S, 8.19; found: C, 53.26; H, 6.37; N, 7.05; O, 24.07; S, 8.07. The solubility of the various salt forms was determined as described [51].

Single-crystal X-ray structure of DMP 450

The bis-hydrochloride was crystallized by evaporation from ethanol/water using a seed crystal from a 1N HCl solution, to yield colorless monoclinic needles. Complete reports including data collection, atomic coordinates, thermal parameters, and interatomic distances and angles are available as supplementary material.

Protease-inhibition assays

Values for the inhibition constant, K_i, were determined with a fluorescent peptide substrate at pH 5.5 with 1.0 M NaCl, as described previously [25]. A polyprotein substrate corresponding to Gag p17 plus the first 78 amino acids of p24 was used to determine the 50 % inhibitory concentration (IC₅₀) for DMP 450 against HIV protease at pH 6.5, 0.15 M NaCl. Inhibition of the aspartyl proteases human renin, porcine pepsin, bovine cathepsin D, and the chymotryptic enzymes human pancreatic chymotrypsin and human neutrophil cathepsin G was measured as previously described [25] using DMP 450 concentrations ranging from 12.5 μM to 1.2 mM.

Antiviral assays

The ability of DMP 450 to inhibit HIV replication in tissue culture was assessed using four different assay systems. The yield of infectious virus produced in acute infections of MT-2 or H9 human T-lymphoid cells and in human peripheral blood mononuclear cells (PBMCs) was measured using a plaque assay as previously described [26]. The antiviral activity of DMP 450 was also determined by measurement of viral RNA accumulation in HIV-1 (RF) acutely infected MT-2 cells as previously described [26,52]. The effect of DMP 450 on production of viral p24 antigen by HIV-infected PBMCs or MT-4 cells was measured using the AIDS Clinical Trials Group/Department of Defense PBMC consensus assay [53] for PBMCs, or the method of Vacca *et al.* for MT-4 cells [10]. The DuPont/NEN p24 ELISA kit was used for p24 quantitation. To determine the influence of plasma protein binding on antiviral potency, human serum albumin (45 mg ml⁻¹) and α-1-acid glycoprotein (1 mg ml⁻¹) were added to tissue culture medium. To demonstrate that the antiviral activity of DMP 450 reflected inhibition of the HIV protease, the effect of DMP 450 on processing of the viral Gag polyprotein was measured by radioimmunoprecipitation of cellular and extracellular viral products from chronically infected Molt-4 human T-lymphoid cells or U937 human monocytoid/macrophage cells as previously described [27]. In each case, the concentration of compound which reduced the measured parameter by 90 % was designated the IC₉₀. The concentration of DMP 450 required to reduce cell viability by 50 % as determined using the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was designated the TC₅₀ [26].

Isolation of HIV-1 from clinical samples

HIV-1 was isolated from patient PBMCs by cocultivation with phytohemagglutinin- and IL-2-stimulated PBMCs from normal donors and resulting virus stocks were titrated using PBMCs as described previously [26,34].

Measurement of antiviral efficacy against mutant variants of HIV-1

The ability of DMP 450 to inhibit the replication of drug-resistant variants of HIV-1 in human T-lymphoid cells or PBMCs was determined in two mutant virus assay systems. Mutant viruses, which were selected *in vitro* by continuous passage in the presence of a symmetrical diol protease inhibitor P9941 [54] or in the presence of the cyclic urea DMP 323 [33], were assessed for their susceptibility to DMP 450 in MT-2 cells using the yield reduction assay. The nucleotide sequence of the protease gene of these mutant viruses was determined.

Each virus is designated by the prefix s, to designate it as a selected virus, and the amino acid position of protease found to be altered. This is preceded and followed by the amino acid present in parental and variant virus, respectively, followed by the designation of the selecting HIV isolate and selecting agent. Thus sVal82Ala(RF/P9941) is a selected drug-resistant variant containing a single amino acid substitution of alanine for valine at position 82 of protease and was derived by tissue culture passage of HIV-1(RF)-infected MT-2 cells against P9941 [54]. The double mutants sVal82Phe/Leu97Val(patient E/DMP 323) and sVal82Phe/Ile84Val(RF/DMP 323) have been previously described [33]. In companion experiments, isogenic viruses with the noted protease-gene alterations in a constant HxB2 background were reconstructed from plasmids that had undergone site-directed mutagenesis to insert specific base-pair changes leading to the desired amino-acid substitutions in the viral protease gene [55]. The reconstructed viruses were assessed for susceptibility towards DMP 450 using the recently adopted Inter-Company Consortium consensus assay, which measures production of viral p24 4 days after acute infection of MT-4 cells [10]. The reconstructed recombinant viruses are named in the same manner as the selected viruses, with the r prefix to indicate their recombinant DNA origin. *In vitro* selection experiments were carried out essentially as described earlier for DMP 323 [33]. Concentrations of DMP 450 were gradually increased over the course of 29 passages. For the HIV designated Donor E, passage 1 contained 0.03 $\mu\text{g ml}^{-1}$ DMP 450; the concentration at passage 29 was 3.0 $\mu\text{g ml}^{-1}$ DMP 450. For HIV H9466, initial DMP 450 concentration was 0.05 $\mu\text{g ml}^{-1}$ and was 3.0 $\mu\text{g ml}^{-1}$ at passage 29. Determination of IC_{50} by yield reduction, and preparation of samples for sequence determination were carried out as previously described [33].

Electron microscopy

Molt-4 and U937 cells were infected with HIV-1(RF) and chronically infected cells derived from extended passage in tissue culture. H9 cells chronically infected with HIV-2(ROD) were produced in similar fashion. Cells were treated with 8.2 μM DMP 450 for 4 days and cells prepared for electron microscopy as previously described [27].

In vivo activity of DMP 450 in murine striated muscle

Autologous plasmids that efficiently express the HIV protease or firefly luciferase genes were constructed using the human cytomegalovirus immediate-early promoter, an SV40 splice site and sequences to ensure polyadenylation. Standard methods were used for large scale plasmid preparation and CsCl purification [56]. Plasmids (in phosphate buffered saline pH 7.2) were injected into the quadriceps muscle of 30 day-old Balb C female mice under Avertin anesthesia. A maximum of 100 μl of solution was injected, containing 10 μg luciferase plasmid with or without 30 μg of HIV protease expression plasmid. Animals were sacrificed after 48 h, the quadriceps muscles removed, weighed and extracted using mechanical homogenization in luciferase assay buffer as described [48,49]. Luciferase activity was determined using a Dynatech Model 1000 luminometer, measuring flash luminescence of 6 μM firefly luciferin (Sigma) containing 1 mM ATP. Mice were dosed with DMP 450 at 10 μg per quadriceps muscle or 100 mg kg^{-1} PO in water 8 h prior to the plasmid injection and BID for 2 days prior to sacrifice and preparation of homogenates for assay. A vehicle of 25:25:50 propylene glycol:polyethylene glycol 400:water was used for DMP 323.

X-ray crystallography of DMP 450–HIV protease inhibitor complexes

Purified recombinant HIV protease mixed with a 10-fold molar excess of inhibitor was crystallized at pH 5.4 using conditions similar to those previously reported [57]. The crystal belongs to the space group $\text{p}6_1$ $a = b = 63.1 \text{ \AA}$, $c = 83.4 \text{ \AA}$. Diffraction data were collected in 2° oscillation images exposed for 90 min using an R-axis imaging plate with an 82 mm crystal-to-detector distance. Intensities for 12478 unique reflections representing 94 % of the possible data to 2.0 Å resolution were obtained from 45866 independent measurements taken on 23 images ($R_{\text{sym}} = 0.071$). The initial R-factor was 0.27 using

atomic coordinates from a model previously refined with other inhibitors of this series [23]. After crystallographic refinement by simulated annealing [58], the R-factor dropped to 0.23. Electron density maps calculated at this stage clearly revealed the bound conformation of the inhibitor. Geometric refinement parameters for the inhibitor were derived from its small molecule structure. The final R-factor is 0.21 for 8433 reflections to 2.0 Å resolution for which $F > 2\sigma(F)$. Coordinates will be deposited in the Brookhaven Data Bank.

Pharmacokinetics studies

The pharmacokinetics of DMP 450 were investigated in the rat, dog, rhesus monkey, chimpanzee and HIV seronegative human volunteers. Dosing was IV or PO. The compound was administered PO in aqueous solution (rat, rhesus, chimpanzee) or as a dry powder in capsules (dog, human). Animals and human subjects were subjected to overnight fast. Dose is expressed as free base equivalents. Compound in plasma samples was isolated by solid-phase extraction, then quantified by HPLC-UV. The absolute oral bioavailability (F%) was determined by comparing the area under the plasma concentration versus time profiles for PO and IV doses, adjusting for differences in dose level. Full details will be published elsewhere (L.S., M.G., *et al.*, unpublished data).

Supplementary material available

Complete reports on the X-ray crystal structure of DMP 450, including data collection, atomic coordinates, thermal parameters and interatomic distances and angles.

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