Design and selection of DMP 850 and DMP 851: the next generation of cyclic urea HIV protease inhibitors

James D Rodgers*, Patrick YS Lam*, Barry L Johnson, Haisheng Wang, Soo S Ko, Steven P Seitz, George L Trainor, Paul S Anderson, Ronald M Klabe, Lee T Bacheler, Beverly Cordova, Sena Garber, Carol Reid, Matthew R Wright, Chong-Hwan Chang and Susan Erickson-Viitanen

Background: Recent clinical trials have demonstrated that HIV protease inhibitors are useful in the treatment of AIDS. It is necessary, however, to use HIV protease inhibitors in combination with other antiviral agents to inhibit the development of resistance. The daunting ability of the virus to rapidly generate resistant mutants suggests that there is an ongoing need for new HIV protease inhibitors with superior pharmacokinetic and efficacy profiles. In our attempts to design and select improved cyclic urea HIV protease inhibitors, we have simultaneously optimized potency, resistance profile, protein binding and oral bioavailability.

Results: We have discovered that nonsymmetrical cyclic ureas containing a 3-aminoindazole P2 group are potent inhibitors of HIV protease with excellent oral bioavailability. Furthermore, the 3-aminoindazole group forms four hydrogen bonds with the enzyme and imparts a good resistance profile. The nonsymmetrical 3-aminoindazoles DMP 850 and DMP 851 were selected as our next generation of cyclic urea HIV protease inhibitors because they achieve 8 h trough blood levels in dog, with a 10 mg/kg dose, at or above the protein-binding-adjusted IC_{90} value for the worst single mutant – that containing the Ile84→Val mutation.

Conclusions: In selecting our next generation of cyclic urea HIV protease inhibitors, we established a rigorous set of criteria designed to maximize chances for a sustained antiviral effect in HIV-infected individuals. As DMP 850 and DMP 851 provide plasma levels of free drug that are sufficient to inhibit wild-type HIV and several mutant forms of HIV, they could show improved ability to decrease viral load for clinically significant time periods. The ultimate success of DMP 850 and DMP 851 in clinical trials might depend on achieving or exceeding the oral bioavailability seen in dog.

Address: DuPont Merck Pharmaceutical Company, E500/4603 Experimental Station, PO Box 80500, Wilmington, DE 19880-0500, USA.

*These authors contributed equally to the work.

Correspondence: James D Rodgers
E-mail: james.d.rodgers@dupontpharma.com

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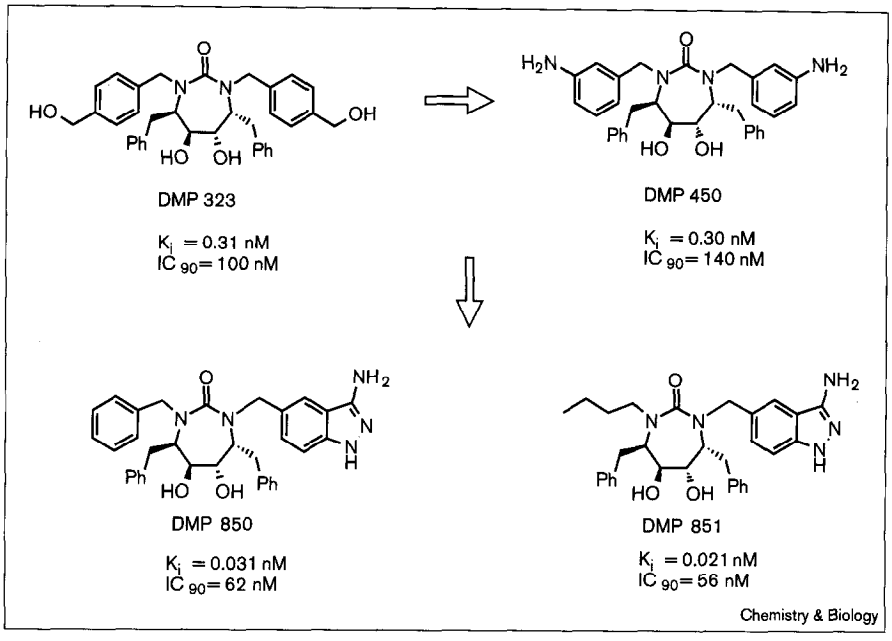
Introduction

AIDS remains a major public health issue in the United States and worldwide. The Center for Disease Control estimates that 1–2 million people in the United States and 22 million people worldwide are infected with the human immunodeficiency virus (HIV), the causative agent of AIDS. Currently, about 450,000 people have died of AIDS or AIDS-related complications in the United States.

Over the past decade, intensive research has provided an insight into the life cycle of HIV. Once the mechanisms for HIV replication were understood, key enzymes were identified, which then became targets for rational drug design. Our research efforts have focused on combating AIDS through inhibition of HIV protease (HIVPR), an essential enzyme for viral propagation [1,2]. Inhibition of HIVPR *in vitro* results in the production of progeny virions that are immature and noninfectious [3,4]. Recent clinical trial results have proven that combination therapy, including a protease inhibitor with a reverse transcriptase inhibitor, provides significant benefit in the treatment of HIV-positive individuals, as measured by surrogate markers and clinical endpoints. The daunting ability of the virus to rapidly generate resistant mutants [5], however, suggests that there is an ongoing need for new HIVPR inhibitors with superior pharmacokinetic and efficacy profiles.

We have reported recently the rational design and discovery of a novel class of nonpeptidal cyclic ureas as potent HIVPR inhibitors [6,7]. The design strategy incorporates the unique structural water molecule commonly found in the X-ray complexes of linear peptidal

Figure 1



Cyclic urea HIV protease inhibitors. Previous clinical candidates DMP 323 and DMP 450, which led to the discovery of current clinical candidates DMP 850 and DMP 851.

mimetic inhibitors and HIVPR in a preorganized cyclic urea scaffold. Structure-based optimization led rapidly to the first clinical candidate, DMP 323 (Figure 1). The clinical trial was terminated, however, because of highly variable oral bioavailability resulting from low aqueous solubility (6 μg/ml) and metabolic instability of the benzylic hydroxymethyl groups. A second clinical candidate, DMP 450 (Figure 1) [8], had good aqueous solubility (130–170 mg/ml) and showed good oral bioavailability in humans. Compared to other protease inhibitors, however, DMP 450 had only a moderate potency and resistance profile. We have therefore continued our efforts to find a compound with a superior profile. This work culminated in the selection of DMP 850 and DMP 851 (Figure 1) as our next generation of cyclic urea HIVPR inhibitors.

Results and discussion

Selection criteria

In our attempts to find a compound superior to DMP 450, we established a rigorous set of selection criteria. Simultaneous optimization of multiple parameters was necessary to achieve our goal. First, we optimized potency against wild-type virus, which is reported as an IC_{90} value. The IC_{90} is the concentration of drug needed to inhibit 90% of viral reproduction in the MT-2 cell-based assay [9].

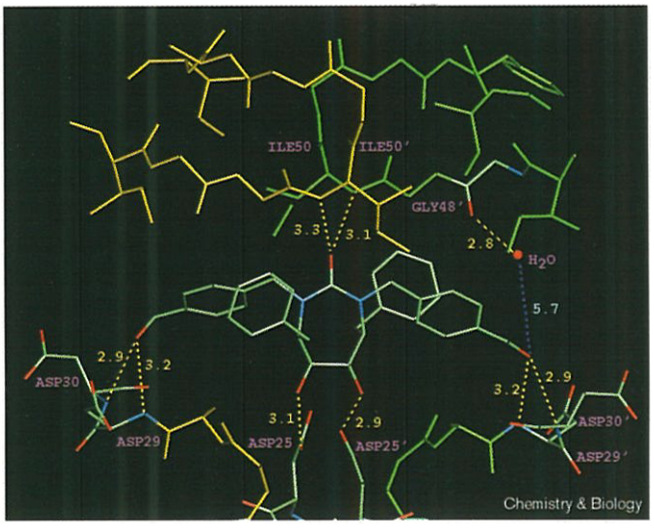
In addition to wild-type potency, it was also important to assess the effect of plasma protein binding on the IC_{90} value [10]. The effect of protein binding was most conveniently screened as a fold shift in the IC_{90} with added human serum albumin (HSA; 45 mg/ml) and human alpha-1-acid glycoprotein (AAG; 1 mg/ml). Once compounds with an attractive overall profile were identified, microdialysis was used for a more accurate measure of protein binding.

Another criterion used to evaluate compounds was the profile to known resistant or mutant virus (the resistance profile). As mentioned above, resistance is the most daunting issue surrounding the discovery of improved HIV protease drugs. Clinically, it has been observed that protease inhibitors must be used in combination with reverse transcriptase inhibitors to inhibit the breakthrough of resistant virus. In attempts to find a drug that effectively inhibits the development of resistance, we screened compounds against a variety of mutant viruses. From selection experiments using DMP 323 and DMP 450, we observed that the worst single mutant for the cyclic urea class contains Ile84→Val (I84V) [11].

Once compounds were assayed for the above criteria, protein-binding-adjusted IC_{90} values for wild type and the I84V mutation were calculated. The protein-binding-adjusted IC_{90} values were calculated by taking the product of the IC_{90} and the fold shift in the wild type IC_{90} with added HSA and AAG.

Finally, compounds were assessed for oral bioavailability and pharmacokinetics in dog with a 10 mg/kg dose. The C_{max} (the maximum plasma concentration) and the time above the protein-binding-adjusted IC_{90} for wild type and the I84V mutant were measured. In order to inhibit the outgrowth of resistant virus, trough blood levels of the drug should meet or exceed the protein-binding-adjusted

Figure 2



X-ray crystal structure of DMP 323 bound to HIV protease. Hydrogen bonds are shown as dashed yellow lines. Distances are in angstroms.

IC_{90} value for the I84V mutant. Our goal was therefore to find a compound that would maintain 8 h trough blood levels in dog, with a 10 mg/kg dose, at or above the protein-binding-adjusted IC_{90} for the worst single mutant — the I84V mutation.

Design

DMP 323 has two metabolically labile *para* hydroxymethyl groups. From the X-ray crystal structure of DMP 323 (Figure 2), we see that the hydroxyl groups form hydrogen bonds to the backbone Asp29 and 30 NH groups located near the S2 pocket. This region of the enzyme is rich in polar hydrogen-bonding residues: Asp29 and Asp30, which have sidechain carboxylate groups, and Gly48, which is hydrogen bonded to a water molecule. As a strategy to improve potency, we designed P2/P2′ groups with functionality oriented to maximize hydrogen-bonding interactions with these polar amino-acid residues.

To improve on the potency and metabolic instability of DMP 323, we replaced the hydroxyl group with stronger hydrogen-bonding functionality and constrained the hydrogen-bonding groups into various benzofused heterocycles [12]. Systematic variation of the hydrogen-bond donor/acceptor functionality led to the discovery of extremely potent antiviral agents. The cyclic urea **1** (Figure 3) with indazole P2/P2′ groups showed a K_i of 18 pM and translated exceptionally well to the cell-based assay with an IC_{90} of 8 nM. Although *in vitro* metabolism studies revealed no significant metabolic liability, the indazole **1** showed low oral bioavailability in dog (F% = 13%, where F% is the ratio of the area under the curve for oral versus intravenous administration of the compound) probably as a result of extremely poor aqueous solubility (3 ng/ml).

In attempts to improve on the oral bioavailability of **1**, we incorporated weakly basic functionality to improve aqueous solubility. To this end, a series of 3-aminoindazoles as P2/P2′ groups was investigated (Table 1) [13]. The parent 3-aminoindazole **2** was an extremely potent inhibitor of HIV protease (K_i < 0.01 nM).

In order to understand the binding motif responsible for the excellent potency of the parent aminoindazole, an X-ray crystal structure of **2** bound to HIV protease was obtained (Figure 4). As was seen in the X-ray structure of DMP 323, the cyclic urea binds symmetrically to the enzyme and the same hydrogen-bond interactions with the urea core are observed. In addition, the indazole N-H of **2** forms a 2.8 Å hydrogen bond to the Asp30 carbonyl group and the nitrogen atom at the two position of **2** forms a 3.2 Å hydrogen bond to the Asp30 N-H. The $-NH_2$ group at the three position forms two hydrogen bonds to the enzyme. There is a 3.0 Å hydrogen bond from the $-NH_2$ group to the Asp29 carboxylate sidechain and a 2.9 Å hydrogen bond to a water molecule, which forms a 2.6 Å hydrogen bond to the Gly48 carbonyl group. These additional hydrogen bonds to Asp29 and Gly48 account for the increased binding of the 3-aminoindazole **2** over the unsubstituted indazole **1**.

In other series of cyclic ureas, we have found that the resistance profile of the drug improves as the number of hydrogen bonds in this region of the enzyme increases

Figure 3

The effect of adding the 3-NH_2 group to indazole **1**.

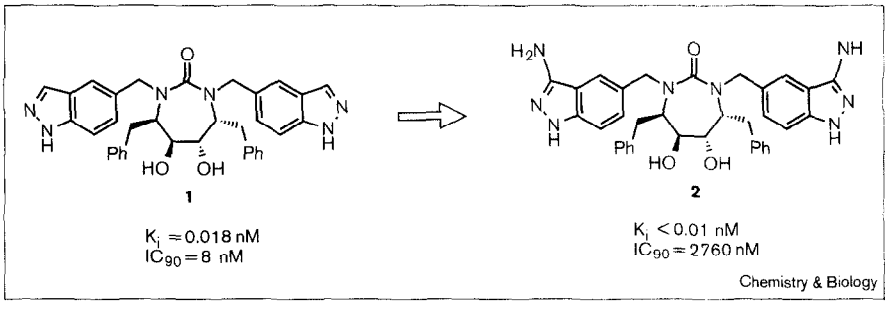
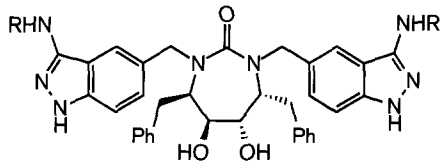


Table 1

Activity and translation of 3-alkylaminoindazole cyclic ureas.



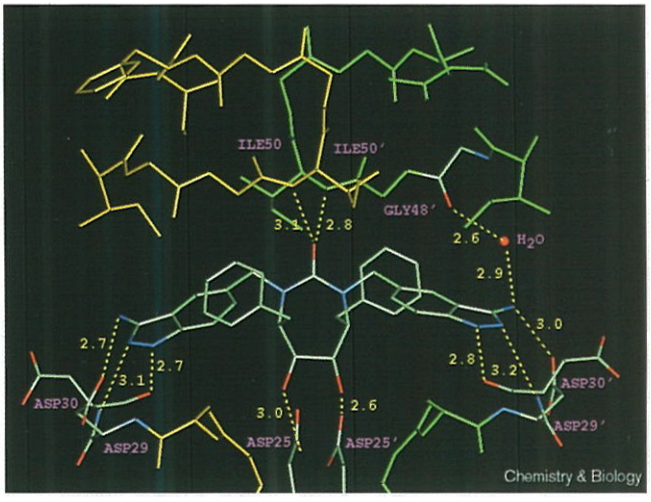
R	K_i (nM)	RNA IC_{90} (nM)	cLogP	$-\log K_i/IC_{90}$
H	<0.01	2760	3.5	>5.4
Me	0.018	279	5.0	4.2
Et	0.041	65	6.0	3.2
i-Pr	0.09	24	6.6	2.4
c-$PrCH_2$–	0.34	38	6.9	2.1
n-Pr	0.23	40	7.0	2.2
–$(CH_2)_4$–	1.30	120	7.9	2.0

[14]. The 3-aminoindazole functionality takes maximum advantage in forming hydrogen bonds and is expected to impart a good resistance profile. This expectation was borne out in resistance testing of **2**, which revealed no loss in antiviral potency (i.e. IC_{90}) against the I84V mutant and only a 1.3-fold loss against the I84V/V82F double mutant (see the section below on activity against mutant variants of HIV-1). By comparison, DMP 450 suffered a sevenfold loss of potency against the I84V mutant and a 100-fold loss against I84V/V82F double mutant [8]. In designing compounds that maximize hydrogen-bonding opportunities, we had achieved a dramatic improvement in the resistance profile of our cyclic urea HIVPR inhibitors.

Unfortunately, the aminoindazole **2** was too polar for good translation of potent enzyme activity to the cell-based assay (IC_{90} = 2760 nM). Translation could be improved by attaching alkyl groups to the 3-NH_2 group of the aminoindazole to increase lipophilicity (Table 1), but a trade-off was seen because increased steric bulk resulted in decreased enzyme potency. The N-ethyl and N-isopropyl analogs had the best IC_{90} values in the series but no measurable blood levels were seen on oral dosing in rat. It is likely that the high molecular weight and large number of hydrogen bond donor/acceptor groups precluded absorption of these analogs.

In order to better address issues surrounding translation and oral bioavailability, we decided to investigate nonsymmetrical cyclic ureas. The idea was to use only one aminoindazole P2 group for optimal binding and then adjust lipophilicity with a simple hydrocarbon P2′ group for good translation. Nonsymmetrical inhibitors have the advantage of reduced molecular weight and fewer polar hydrogen-bonding groups. In addition, making the compounds nonsymmetrical should also break up crystal packing for better solubility and oral bioavailability. This hypothesis was supported by the single crystal X-ray structure of

Figure 4



X-ray crystal structure of 3-aminoindazole **2** bound to HIV protease. Hydrogen bonds are shown as dashed yellow lines. Distances are in angstroms.

DMP 323, which showed a network of intermolecular hydrogen bonds between the P2/P2′ hydroxymethyl groups and the urea carbonyl and diol functionality of adjacent molecules. This network of hydrogen bonds can be partially disrupted with a hydrocarbon P2′ group. It was proposed that crystal packing could be decreased further using a floppy alkyl P2′ group.

Chemistry

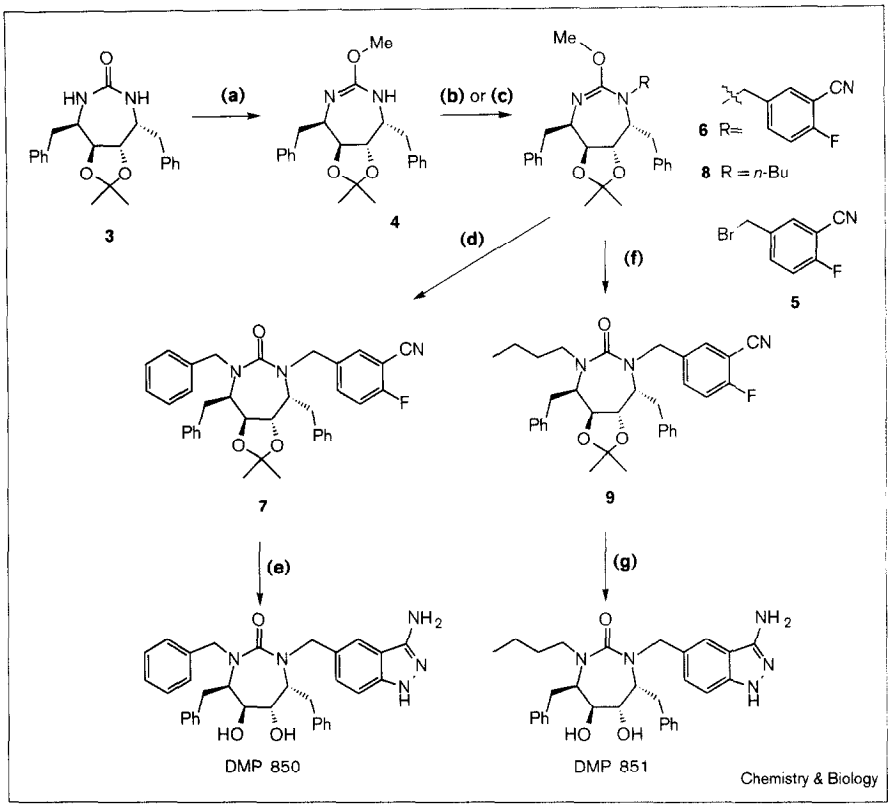
An efficient and general synthesis of nonsymmetrical cyclic ureas was developed (Figure 5). Alkylation of urea **3** [15] with methyl triflate gave the isourea **4** in 75% yield. Alkylation of **4** with benzyl bromide **5** afforded mono-alkylated isourea **6** (92% yield). Condensation of **6** with benzyl bromide in refluxing acetonitrile yielded the nonsymmetrical urea **7** (95% yield). Alternatively, compounds containing alkyl P2 groups were synthesized by first attaching the alkyl substituent to the isourea with subsequent condensation with **5**. Construction of the aminoindazole ring was accomplished in quantitative yield by refluxing with hydrazine. Deprotection with mild acid then afforded the target compounds. This route proved very general, efficient and high yielding. The compounds in Table 2 were prepared by this route to investigate if nonsymmetrical cyclic ureas would have improved characteristics. In designing nonsymmetrical analogs, the molecular weight was decreased below ~620 and three hydrogen-bonding groups were eliminated. In addition, the crystallinity of the nonsymmetrical analogs was lower, as shown by improved solubility characteristics.

Inhibition of HIV protease

Although nonsymmetrical compounds were attractive for reasons already mentioned, it was uncertain if one hydrogen-bonding P2 group would impart sufficient potency. As

Figure 5

The synthesis of DMP 850 and DMP 851. Conditions: **(a)** MeOtf, DCE, reflux. **(b)** NaH, DMF, **5**. (c) NaH, DMF, *n*-BuI. **(d)** PhCH2Br, CH3CN, reflux. **(e)** hydrazine, n-BuOH, reflux, 3 N HCl, THF. **(f)** 5, CH3CN, reflux. **(g** hydrazine, *n*-BuOH, reflux; HCl, MeOH.



seen from Table 2, one aminoindazole group was adequate to give very potent inhibitors with K_i values of less than 30 pM. This is the same level of potency obtained with the symmetrical methyl (K_i = 18 pM) and ethyl (K_i = 41 pM) substituted 3-aminoindazoles. Similar potency was achieved with three fewer hydrogen-bonding groups and molecular weights were reduced by over 100 mass units.

Also included in Table 2 are assay values for indinavir, ritonavir, saquinavir, nelfinavir and VX-478. Against peptide substrate in a sensitive fluorescence-based assay [16], the cyclic urea analogs are considerably more potent than the approved drugs and other competitor compounds. In the viral polyprotein-processing assay, the cyclic urea inhibitors are comparable to the benchmark compounds tested. Differences in the apparent potency for the two assays are attributed to differences in substrate, pH, ionic strength, and ratio of substrate and inhibitor to enzyme. For compounds with high (< 1 nM) affinity for the enzyme, titration behavior is observed, and IC_{50} values approach enzyme concentration.

Inhibition of HIV replication

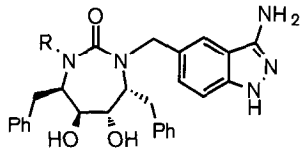
In addition to maintaining potency against HIVPR, translation of enzyme activity to antiviral activity was achieved easily through manipulation of the lipophilicity of the hydrocarbon P2′ substituent (Table 3). Although the cyclopropylmethyl analog was too polar for good translation (IC_{90} = 114 nM), the cyclobutylmethyl analog translated well to give an IC_{90} of 18 nM. Similarly, the benzyl (DMP 850), butyl (DMP 851) and naphthyl analogs all translated well to give IC_{90} values of 62, 56 and 23 nM, respectively. As was found for enzyme activity, antiviral potency equivalent to the best symmetrical aminoindazole analogs was obtained.

It is interesting to note that translation with the nonsymmetrical analogs was obtained with one log unit lower clogP than analogous symmetrical compounds. This result was probably a result of the decreased number of hydrogen-bonding groups lowering the energy of desolvation for better membrane permeability. Having good translation of enzyme activity to antiviral activity with more polar analogs that have increased aqueous solubility increased the likelihood for better oral bioavailability.

To further assess the antiviral potency of DMP 850 and DMP 851, we measured the concentrations required for 90% inhibition using three different types of antiviral assays and a variety of cell types and HIV isolates (Table 4). DMP 850 and DMP 851 were potent against laboratory strains of HIV-1 and HIV-2 as well as against primary clinical isolates derived from zidovudine-resistant

Table 2

Potency of cyclic ureas in two enzyme inhibition assays.



Cyclic urea (R)	K_i (nM ± sd)*	GAG IC_{50} (nM ± sd)†
Cyclopropylmethyl	0.02	25
Cyclobutylmethyl	0.016	7.8
2-Naphthylmethyl	0.023	12
Benzyl (DMP 850)	0.031 ± 0.021 (n=5)	49.0 ± 24.2 (n=4)
n-Butyl (DMP 851)	0.021 ± 0.013 (n=4)	39.5 ± 24.4 (n=4)
n-Pentyl	0.041	41
n-Hexyl	0.13	130
Indinavir	0.370 ± 0.04 (n=3)	20.0 (single value)
Saquinavir	0.150 ± 0.07 (n=3)	13.1 ± 5.2 (n=2)
Nelfinavir	0.530 ± 0.18 (n=2)	nd
VX-478	0.170 ± 0.06 (n=2)	13 (single value)
Ritonavir	0.370 ± 0.06 (n=2)	nd

*Determined using fluorescent peptide substrate at pH 5.5.
†Determined using GAG polyprotein at pH 6.5. nd, not determined

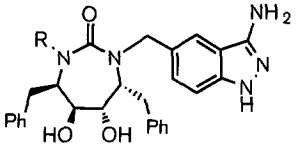
patient samples (A018C, E, WR 10983) [17]. The average potency observed against laboratory strains versus clinical isolates is summarized in Table 5. The values from the RNA-based assay utilizing the RF virus and MT-2 cells for DMP 850 and DMP 851, as well as the four approved HIV protease inhibitors (indinavir, ritonavir, saquinavir and nelfinavir) and one experimental agent (VX-478) are shown to allow direct comparison. In addition, data for potency against clinical isolates using published values for competitor compounds are summarized. As noted in Table 5, the antiviral activity of DMP 850 or DMP 851 against the RF strain is comparable to VX-478 and ritonavir, and about half as potent as indinavir or nelfinavir. Saquinavir is more potent than all of the other inhibitors examined. Against clinical isolates, the potencies of DMP 850 and DMP 851 are within the broad range of values reported for indinavir, ritonavir, saquinavir and nelfinavir. VX-478 is more potent than the other compounds examined against clinical isolates.

Pharmacokinetics

Although translation to antiviral activity was encouraging, the best attribute of the nonsymmetrical analogs was their outstanding oral bioavailability. The pharmacokinetic profile of the cyclic ureas was examined in dogs. Animals were dosed in cosolvent vehicle for intravenous administration, and in cosolvent or as neat powder in a capsule for oral administrations. In Table 3 we see that the cyclobutyl analog showed 100% oral bioavailability with a C_{max} of 8.9 μM and a 2.6 h half life. Although the benzyl analog,

Table 3

Antiviral activity and oral bioavailability of cyclic ureas.



R	IC_{90} (nM)*	clog P	C_{max} (μM)†	$t_{1/2}$ (h)	F%
Cyclopropylmethyl	114	4.6	nd		
Cyclobutylmethyl	18	5.1	8.9	2.6	100
Benzyl (DMP 850)	62	5.2	9.6	3.5	60
n-Butyl (DMP 851)	56	5.2	11.7	7.7	57
n-Pentyl	203	5.71	nd		
n-Hexyl	756	6.24	nd		
2-Naphthylmethyl	23	6.3	4.7	nd	nd

*See [9]. †Determined in dog using a 10 mg/kg dose. nd, not determined.

DMP 850, showed a higher C_{max} (9.6 μM) and a 3.5 h half life, the best oral bioavailability was seen with the butyl analog, DMP 851, which showed a 11.7 μM C_{max} and 7.7 h half life. The butyl analog is the lowest molecular weight analog of this group (MW = 528) and the naphthyl analog (MW = 612) is the highest molecular weight cyclic urea that still has good oral bioavailability. In making the compounds nonsymmetrical, we have decreased the molecular weight and increased aqueous solubility to give compounds with outstanding oral bioavailability.

An interesting relationship between the C_{max} in dog and the molecular weight of our cyclic ureas is shown in Figure 6. Only compounds with molecular weights less

Table 4

Potency of DMP 850 and DMP 851 against laboratory isolates.

Virus	Cells	Assay	DMP 850 IC_{90} (nM)	DMP 851 IC_{90} (nM)
RF	MT-2	RNA	61.9 ± 15.8 (n=15)	56.2 ± 12.8 (n=13)
RF	PBMC	p24	157	167
RF	MT-2	Yield	60.5 ± 47.6 (n=11)	44.3 ± 10.9 (n=4)
RF	MT-4	p24	107 ± 25.2 (n=2)	130 ± 25.4 (n=2)
HxB2	MT-4	p24	92.3 ± 21.0 (n=13)	68.5 ± 14.7 (n=7)
HXB2	PBMC	p24	153	96.6
HIV-2 (Rod)	MT-2	Yield	52.7	25.0
A018C	PBMC	p24	155	142
Thai 9466	PBMC	p24	153	116
Thai H9156	MT-2	p24	94.9 ± 46.7 (n=2)	53.0 ± 6.6 (n=2)
'WR 10983	PBMC	p24	173	174
E	MT-2	p24	174 ± 49 (n=2)	84.6 ± 25.4 (n=2)

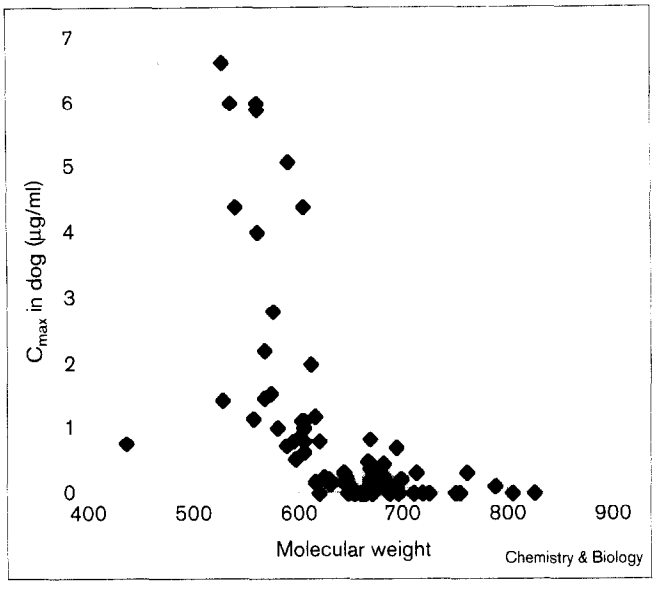
Table 5

Comparison of antiviral potencies.

Inhibitor	RF virus	Clinical isolate
	RNA IC_{90} (nM)	IC_{90} (nM)
DMP 850	61.9	149
DMP 851	56.2	114
Indinavir	31.9	25–100*
Ritonavir	45.8	3.8–153†
Saquinavir	5.6	0.3–200‡
Nelfinavir	20.1	7–196§
VX-478	41.0	12–19#

*Crixivan™ package insert and Vacca *et al.* [27]. Values correspond to 95% inhibition, range is for all isolates examined, including clinical isolates and laboratory strains. †Norvir™ package insert and Kempf *et al.* [28]. Values correspond to 50% inhibition, range is for all isolates examined, including clinical isolates and laboratory strains. ‡Invirase™ package insert and Roberts [29]. Values correspond to 90% inhibition, range is for all isolates examined, including clinical isolates and laboratory strains. §Shetty *et al.* [30]. Values correspond to 95% inhibition, range is for all isolates examined, including clinical isolates and laboratory strains. #Values correspond to 50% inhibition. Range is for AZT-sensitive and AZT-resistant clinical isolates.

Figure 6



Peak plasma levels versus molecular weight of cyclic ureas.

than ~620 have good oral bioavailability. Above ~620 MW there is a steep drop-off in C_{max}. All cyclic ureas above 620 MW had a C_{max} of less than 1 μM. Our most potent analogs with the best resistance profiles are above the 620 MW cut-off [14]. It has been necessary to compromise somewhat on potency and resistance profile to obtain compounds with good oral bioavailability.

Protein binding

After discovering this series of highly bioavailable non-symmetrical aminoindazoles, we determined the effect of protein binding and the profile against a variety of resistant mutant viruses. Many drugs bind to plasma proteins [18,19]. The effect of such binding is to decrease the concentration of free drug available to penetrate cells, and bind to intracellular targets. We thus explored the effects of the two major components of human plasma, namely, HSA and AAG on antiviral potency. The IC_{90} value was determined by measuring viral RNA accumulation in MT-2 cells infected with HIV-1(RF) using the RNA assay. The potency of the compounds observed in tissue culture supplemented with concentrations of HSA and AAG likely to be presented in the plasma of HIV-infected individuals, namely 45 mg/ml HSA and 1 mg/ml AAG, is shown in Table 6. The cyclobutylmethyl analog showed very high protein binding with a > 20-fold increase in the IC_{90} with HSA and AAG present. This gives a protein-binding-adjusted IC_{90} of greater than 0.36 μM, which is probably exceeded by the 8 h trough level of 2.7 μM. The benzyl analog (DMP 850) showed only an eightfold loss with protein binding to give an adjusted IC_{90} of 0.49 μM, which is exceeded by the trough level of 2.8 μM. The butyl analog (DMP 851) showed higher protein binding with a 17-fold shift and a 0.89 μM protein-binding-adjusted IC_{90}. The outstanding pharmacokinetics of the butyl analog, however, gives a trough level of 7.2 μM, which is well above the adjusted IC_{90}. The naphthyl analog has the lowest adjusted IC_{90} of 0.23 μM, which is also covered by the trough level of 0.7 μM. As all of the analogs maintain 8 h trough levels above their protein-binding-adjusted IC_{90} values, they should be effective at inhibiting wild-type virus in the clinic, provided similar pharmacokinetics are seen in humans as in dog.

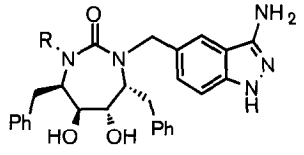
Binding to plasma proteins is substantial for other HIV protease inhibitors as well. We have observed that the concentration required for 90% inhibition of replication in the presence of HSA and AAG is substantially increased for ritonavir (38.7-fold), nelfinavir (32.4-fold) and VX-478 (29.4-fold) compared to tissue culture medium. Only indinavir shows a modest shift, 1.9-fold, consistent with its moderate binding (60%) to human plasma, demonstrated using physical separation methods.

Activity against mutant variants of HIV-1

The final parameter to be considered was resistance. Because of the high error rate of the viral reverse transcriptase, coupled with rapid turnover of large numbers of virions each day, the HIV-infected individual harbors a mixture of HIV species, including potential mutant forms with amino-acid changes in the protease [20,21]. The proportion of potential mutant HIV relative to wild type is a complex function of the relative 'fitness' of mutants. In the presence of an agent that shows lowered ability to inhibit mutant virus relative to wild-type virus, selective

Table 6

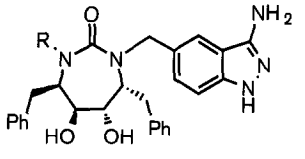
Effect of protein binding on antiviral potency.



R	Wild type IC_{90} (μM)	Protein-binding shift	Protein-binding-adjusted IC_{90} (μM)	8 h trough (μM)
Cyclobutylmethyl	0.018	> 20×	>0.36	2.7
2-Naphthylmethyl	0.023	10×	0.23	0.7
Benzyl (DMP 850)	0.062	8×	0.49 ± 0.13	2.8
n-Butyl (DMP 851)	0.056	17×	0.89 ± 0.25	7.2

Table 7

Antiviral potency against the I84V mutant.



R	IC_{90} sI84V (μM)	Fold increase over WT	Protein-binding shift	Pb-adjusted IC_{90} sI84V (μM)	8 h trough (μM)
Cyclobutylmethyl	0.53	8	>20×	>11	2.7
2-Naphthylmethyl	0.43	9	10×	4.3	0.7
Benzyl (DMP 850)	0.37 ± 0.16	6	8×	3.1 ± 1.4	2.8
n-Butyl (DMP 851)	0.39 ± 0.67	8	17×	6.6 ± 1.1	7.2

pressure can result in a relative outgrowth of mutant populations. In addition to the potential for pre-existing populations of virus, mutations can arise *de novo* from wild-type HIV if inhibition of replication is incomplete. The appearance of mutant forms of HIV in the presence of suboptimal levels of HIV protease inhibitors has been demonstrated both with tissue-culture-based selection experiments [8,22] and in patients undergoing therapy [23,24]. Thus, consideration of the potency of a potential new therapeutic agent against mutant forms of virus likely to pre-exist in the quasi species is crucial.

As mentioned earlier, selection experiments have shown that the worst single mutant for the cyclic ureas is I84V. In order to have an extended antiviral effect, plasma levels should meet or exceed the protein-binding-adjusted IC_{90} value for the I84V mutant. As shown in Table 7, the cyclobutylmethyl analog showed an eightfold increase in the IC_{90} against the I84V mutant. This gives a protein-binding-adjusted IC_{90} of greater than 11 μM, which is not covered by the C_{min} of 2.7 μM. The naphthyl analog showed a ninefold shift and a 4.3 μM protein-binding-adjusted IC_{90}, which is not covered by the 0.7 μM trough level. The benzyl (DMP 850) and butyl (DMP 851) analogs showed only a sixfold and eightfold loss, respectively, however, to give protein-binding-adjusted IC_{90} values of 3.1 ± 1.4 μM and 6.6 ± 1.1 μM, which are both covered (within error) by the trough levels of 2.8 and 7.2 μM. As trough levels of DMP 850 and DMP 851 met or exceeded the protein-binding-adjusted IC_{90} value for the worst single mutant, these compounds were both selected as the next generation of development compounds.

In order to further assess the resistance profile of DMP 850 and DMP 851, we measured the potency against a range of mutant HIVs (Table 8). Mutant HIVs for examination were from two sources. Previous selection experiments with DMP 323 had resulted in viruses carrying the single mutation (in the protease gene) of I84V, the single mutation of V82F or the double mutation V82F/I84V [11]. I84V has also been observed as a predominant mutation in *in vitro* selection experiments with DMP 450 [8]. It is important to note that, in addition to the mutations at position 82 and 84 in the protease gene of the selected virus, we have found that there are also compensatory mutations within the Gag p17 (matrix) protein in this virus [25]. At least one of these p17 mutations is required for viable virus; the selected 'double' virus is thus, at least, a triple mutant relative to the wild type, and is designated sV82F/I84V/Gag p17. Ongoing experiments with a carboxy-amidobenzyl-substituted cyclic urea [14] have yielded a virus carrying the mutations A71V and K41I. We have also selected a mutant virus carrying three mutations in the reverse transcriptase gene in the presence of the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (DMP 266) [17]. These viruses are designated with the 's' prefix to indicated tissue-culture-selected viruses. Potency against selected mutant viruses was determined by yield reduction, except for the DMP 266-resistant virus where p24 measurement was used. In addition, recombinant viruses (designated by the 'r' prefix) were constructed carrying single mutations corresponding to V82F, I84V, D30N or V82A, or carrying double mutations V82A/I84V, G48V/L90M or V32I/V82I. The G48V/L90M virus has been reported in conjunction with the development of resistance to saquinavir. We also constructed recombinant virus carrying the triple mutation M46I/I47V/I50V, which has been associated with resistance to VX-478. Also constructed was the virus described as arising in selection procedures to ritonavir, carrying five mutations (M46I/L63P/A71V/V82F/I84V), and the virus described in patient samples undergoing suboptimal indinavir therapy, and carrying five mutations (L10R/M46I/L63P/V82T/I84V). Potency against recombinant viruses was determined by p24 measurement. Table 8 shows the potency of DMP 850 and DMP 851 towards these viruses. The RF strain of HIV-1 and the wild-type recombinant virus HXB2 are shown for

Table 8

Potency of DMP 850 and DMP 851 against mutant HIV.

Virus	DMP 850 potency (nM)	Fold increase relative to WT	DMP 851 potency (nM)	Fold increase relative to WT
RF	61 ± 48	1.0	47 ± 11	1.0
sV82F	250	4.1	152	3.2
sI84V	370 ± 160	6.0	388 ± 67	8.3
sV82F/I84V/Gag p17	2100 ± 1000	34	1790 ± 13	38
sA71V/K41I	166	2.7	176	3.7
HXB2	92 ± 21	1.0	69 ± 15	1.0
rV82A	135 ± 46	1.5	nd	nd
rV82F	124 ± 54	1.3	130 ± 25	1.9
rI84V	273 ± 116	3.0	227 ± 80	3.3
rD30N	nd		17	0.3
rV82A/I84V	564 ± 341	6.1	nd	nd
rV32I/V82I	192	2.1	nd	nd
G48V/L90M	50 ± 7.5	0.5	53	0.8
VX-triple*	257 ± 70	2.8	87 ± 40	1.3
Merck†	1410 ± 920	15.4	928	13
Abbott‡	1520 ± 640	16.6	1470 ± 230	21
DMP266§	103 ± 53	1.7	62.5 ± 5.7	nd

*Corresponds to the triple mutant associated with resistance to VX-478, containing three mutations (M46I/I47V/I50V). †Corresponds to the quintuple mutant resistant to Crixivan™, containing five mutations (L10R/M46I/L63P/V82T/I84V). ‡Corresponds to the quintuple mutant resistant to Norvir™, containing five mutations (M46I/L63P/A71V/V82F/I84V). §Corresponds to the triple RT mutant selected for by the NNRTI DMP 266, carrying the mutations V179D, L100I, Y181C in the reverse transcriptase. DMP 266 is 1000-fold less sensitive to this virus than to wild-type HIV. WT, wild type; nd, not determined.

comparison, and the fold reduction for the mutants relative to these wild-type viruses is shown.

Both DMP 850 and DMP 851 retain potency against simple mutant variants including V82F, I84V, A71V, D30N and V82A. The largest loss in potency for a single mutant variant is noted for the selected I84V virus. The fold increase in IC_{90} value for this virus is greater than for the pure recombinant virus carrying the same mutation. This apparent influence of background (RF versus HXB2) has been observed with other protease inhibitors [26] and might be related to additional compensatory mutations in the gag or pol genes in the selected virus. In addition, DMP 850 and DMP 851 retain potency against the D30N mutant selected for *in vivo* by nelfinavir [24], the double mutant known to be resistant to saquinavir, and the triple mutant known to be resistant to VX-478. Both DMP 850 and DMP 851 are fully active against a triple mutant variant that is highly resistant to the NNRTI DMP 266. Thus, it is expected that DMP 850 and DMP 851 would be effective in patients previously treated with these agents, even if resistance and treatment failure had occurred. The largest losses in potency of DMP 850 and DMP 851 occur against multiply mutant viruses that carry mutations in both amino acids 82 and 84. These mutations occupy the S1/S1′ pocket and overlap with the S2/S2′ pocket in the protease dimer. These viruses include the five-amino-acid variant selected for by indinavir, the five-amino-acid mutant virus selected for by ritonavir and the three-amino-acid mutant virus selected for by DMP 323. Thus, three or more mutations appear to be required for significant (tenfold or more) loss of sensitivity to these nonsymmetrical cyclic ureas. As these multiply mutant viruses have been shown by a variety of enzymatic assays to be defective in their overall vitality, it is unlikely that significant levels of such crippled virus pre-exists in the patient prior to treatment.

To directly identify variants of HIV that might arise under selective pressure by DMP 850 or DMP 851, two different isolates of HIV-1 (Thai H9466, a wild-type clinical isolate, and E, a ZDV-resistant clinical isolate) were passaged in the presence of gradually increasing concentrations of DMP 850 or DMP 851 in MT-2 cells. We previously used identical conditions to characterize the emergence of DMP 450-resistant variants, and found that significant loss of sensitivity occurred within 29 passages, and was associated with the presence of two to five mutations, including I84V, V82I, D60E, L10F, M46L or M46I and K45I [8]. Additional experiments were also conducted with PBMCs infected with the laboratory strain RF, and exposed to constant concentrations of DMP 850 or DMP 851 for periods of up to 50 weeks. Table 9 shows the results of *in vitro* selection experiments for DMP 850 and DMP 851. The data suggest that resistance to DMP 850 and DMP 851 develops more slowly than for previous cyclic ureas and is associated with the sequential accumulation of mutations. Importantly, the I84V mutation features prominently in those viruses, showing phenotypic resistance as measured by increases in IC_{90} value. After 50 weeks exposure to 57 nM DMP 851, the virus recovered was threefold resistant and remained wild type within the protease gene. After 40 weeks exposure to DMP 850, virus with mutation of V82A was observed, which was eightfold resistant. Ten additional weeks of exposure to DMP 850 did not lead to the domination of the V82A mutant.

Significance

In selecting our next generation of cyclic urea HIV protease inhibitors, we established a rigorous set of criteria designed to maximize chances for a sustained antiviral effect in HIV-infected individuals. It has been extremely difficult to find compounds that have sufficient oral bioavailability to provide adequate free drug levels to inhibit wild-type and mutant viral strains. Compromises in potency and resistance profile were necessary to obtain compounds with good oral bioavailability. Although many compounds in our program were extremely potent antiviral agents with remarkably flat resistance profiles, it was

Table 9

***In vitro* selection of DMP 850 and DMP 851 mutants.**

Compound	Virus	Passage number	IC_{90} (nM)	Fold increase	Mutations observed
DMP 450	E	0	89	Control	
		18	912	10	M46L, V82V+I, I84V+I
		29	4050	45	K45K+I,M46L,V82V+I I84V,L90L+M
DMP 850	E	0	78	Control	
		16	>178	>2.3	ND
		25	687	8.8	M46L/V82A/I84V
		35	2460	32	L10F/L33F/M46L/ V82A/I84V
		46	10100	130	ND
DMP 851	E	0	75	Control	
		17	152	2.0	
		25	386	5.1	
		48	11250	150	
DMP 450	Thai	0	130	Control	
		12	1260	10	D60E/I84V
		29	7780	60	L10F/M46I/D60E/I84V
DMP 850	Thai	0	63.5	Control	
		16	460	7.2	
		25	471	7.4	
		36			
DMP 851	Thai	0	74	Control	
		18	201	2.7	
		25	563	7.6	K45I/I84V
		36	3010	41	K45I/V82I/I84V
		48		120	

the outstanding oral bioavailability and pharmacokinetics of the nonsymmetrical, aminoindazole-containing cyclic ureas that prevailed and resulted in the selection of DMP 850 and DMP 851 as our next generation of clinical candidates. The ultimate success of DMP 850 and DMP 851 in clinical trials might depend on achieving or exceeding the oral bioavailability seen in dog.

Materials and methods

Synthesis of DMP 850

Alkylation of urea ***3***. To a suspension of the urea (10.0 g; 27.3 mmol) in dichloroethane (100 ml) was added methyltriflate (3.4 ml; 30 mmol). After refluxing overnight, the reaction was washed with sat. $NaHCO_3$, sat. NaCl, dried (Na_2SO_4) and evaporated leaving 12.5 g of yellow oil. Column chromatography (flash SiO_2; 25% EtOAc/hexane) gave 7.86 g of **4** as a pale yellow oil which crystallized on standing (75% yield). ^1H NMR (300 MHz, $CDCl_3$, ppm): 1.52 (s, 6H); 2.60-2.77 (m, 2H); 3.09 (t, 2H); 3.45 (s, 3H); 3.48-3.55 (m, 1H); 4.12-4.22 (m, 2H); 4.30-4.38 (q, 2H); 7.15-7.35 (m, 10H). m.p. = 97°-100°C. $MH^+ = 381$.

Synthesis of isourea ***6***. To a 0°C solution of the isourea **4** (4.43 g; 11.7 mmol) and bromide **5** (5.00 g; 23.3 mmol) in DMF (50 ml), was added NaH (60% in mineral oil; 1.40 g; 35.0 mmol). After warming to room temperature and stirring overnight, the reaction was added to 25% Et_2O/EtOAc. The organic phase was washed with water (3×), saturated NaCl, dried (Na_2SO_4) and evaporated leaving a yellow oil. Column chromatography (flash SiO_2; 25% EtOAc/hexane) gave 5.55 g of isourea **6** as a colorless oil (92% yield). ^1H NMR (300 MHz, $CDCl_3$, ppm): 1.48 (s, 3H); 1.50 (s, 3H); 2.68 (dd, 1H); 2.78 (dd, 1H); 3.02-3.11 (m, 2H); 3.25 (d, 1H); 3.22 (s, 3H); 3.57-3.65 (m, 1H); 4.09-4.23 (m, 3H); 4.23 (d, 1H); 6.95-7.40 (m, 13H). $MH^+ = 514$.

Synthesis of urea ***7***. A solution of the isourea **6** (2.78 g; 5.41 mmol) and benzyl bromide (1.93 ml; 16.2 mmol) in acetonitrile (15 ml) was refluxed overnight. The reaction was evaporated and subjected to column chromatography (flash SiO_2; 20% EtOAc/hexane) to give 3.02 g of **7** as a white foam (95% yield). ^1H NMR (300 MHz, $CDCl_3$, ppm): 1.40 (s, 3H); 1.45 (s, 3H); 2.70-3.07 (m, 5H); 3.52 (d, 1H); 3.68-3.88 (m, 3H); 3.97 (dd, 1H); 4.53 (d, 1H); 4.90 (d, 1H); 6.95-7.46 (m, 18H). $MH^+ = 590$.

Conversion of nitrile ***7*** *to DMP 850*. A solution of the nitrile 7 (3.02 g; 5.13 mmol) in n-BuOH (30 ml) and hydrazine hydrate (6 ml) was refluxed overnight. The reaction was added to EtOAc and washed with 10% citric acid (2×), sat. NaCl, sat. $NaHCO_3$, sat. NaCl, dried ($NaSO_4$) and evaporated leaving 3.09 g of a white foam (100% yield) which was used in the next step without purification. The foam was dissolved in 3N HCl (10 ml) and THF (40 ml) and stirred overnight. The reaction was added to EtOAc and washed with sat. $NaHCO_3$, sat. NaCl, dried ($NaSO_4$) and evaporated leaving an orange oil. Column chromatography (flash SiO_2, 7% MeOH/CH_2Cl_2 and 0.8% NH_4OH) gave 2.15 g pink glass. Crystallization from 3:1 CH_2Cl_2/ Et_2O gave 1.7 g of DMP 850 as pale pink crystals, which were dried overnight under high vacuum at 85°C. ^1H NMR (300 MHz, DMSO, ppm): 2.75-3.00 (m, 5H); 3.30-3.60 (m, 5H); 4.72 (d, 1H); 4.75 (d, 1H); 5.01 (d, 2H), 5.32 (br s, 2H); 6.98-7.40 (m, 18H); 11.33 (br s, 1H). mp = 134°-139°C. $MH^+ = 562.3$.

Synthesis of DMP 851

Synthesis of isourea ***8***. To a solution of **4** (10.0 g, 26.3 mmol) in anhydrous DMF (30 ml) was added sodium hydride (1.58 g, 65.8 mmol). The reaction mixture was stirred at room temperature for 45 min followed by dropwise addition of a solution of 1-iodobutane (9.68 g, 52.6 mmol) in anhydrous DMF (10 ml). After the addition, the stirring was continued at room temperature overnight. The reaction mixture was cooled to 0°C and methanol (5 ml) was added to quench excess sodium hydride. The mixture was partitioned between ethyl acetate (200 ml) and water

(150 ml). The organic phase was separated and washed with water (4 × 100 ml), brine (100 ml) and dried over sodium sulfate. Flash chromatographic purification (25% EtoAc/Hex.) gave n-butyl isourea **8** (10.5 g, (92% yield): MS(NH_3-CI/DDIP) (M+H$^+$)437.2(100%); ^{1}H NMR(300 MHz, $CDCl_3$, 25°C) d 7.23 (m, 10H), 4.19 (m, 3H), 3.64 (m, 1H), 3.44 (s, 3H), 3.36 (m, 1H), 3.02 (m, 2H), 2.76 (m, 2H), 2.04 (m, 1H), 1.52 (s, 3H), 1.49 (s, 3H), 1.21 (m, 4H), 0.82 (t, J = 7.0 Hz, 3H).

Alkylation of isourea ***8***. To a solution of **8** (5.0 g, 11.5 mmol) in acetonitrile (40 ml) was added 4-fluoro-3-cyanobenzyl bromide **5** (3.68 g, 17.25 mmol). The reaction mixture was refluxed overnight. After the solvent was removed under reduced pressure, the residue was purified using flash chromatography (35% EtOAc/hexane) to give cyclic urea **9** as a white solid (4.5 g, 71% yield): MS(NH_3-CI/DDIP) (M+H$^+$)556.3(100%); ^{1}H NMR(300 MHz, $CDCl_3$, 25°C) d 7.41 (m, 1H), 7.28 (m, 7H), 7.13 (d, J = 9.2Hz, 2H), 7.05 (t, J = 8.8 Hz, 1H), 6.95 (d, J = 9.2 Hz, 2H), 4.50 (d, J = 14.0 Hz, 1H), 4.07 (m, 2H), 3.70 (m, 3H), 3.44 (t, J = 7.7 Hz, 1H), 2.90 (m, 4H), 2.12 (m, 1H), 1.50 (s, 6H), 1.26 (m, 4H), 0.83 (t, J = 7.0 Hz, 3H).

Conversion of ***9*** *to DMP 851*. To a solution of **9** (4.5 g, 8.11 mmol) in *n*-butanol (20 ml) was added hydrazine hydrate (0.81 g, 16.2 mmol). The mixture was refluxed for 6 h. The solvent and excess hydrazine were removed under reduced pressure. The residue was dissolved in anhydrous methanol (20 ml) followed by the addition of 4 M HCl in dioxane (2 ml). The reaction mixture was stirred at room temperature for 2 h. Methanol was removed and the residue was partitioned between ethyl acetate (80 ml) and sodium bicarbonate (sat.) (50 ml). The organic phase was separated, washed with water (2 × 50 ml) and dried over sodium sulfate (anhydrous). Flash chromatographic purification gave DMP 851 (3.0 g, 72% yield.) as a white solid: MP 129-131°C; MS(NH_3-CI/DDIP) (M+H$^+$) 528.3(100%); HRMS calc'd for $C_{31}H_{37}N_5O_3$+1 528.2975, found 528.2958; ^{1}H NMR(300 MHz, CD_3OD, 25°C) d 7.19 (m, 12H), 6.98 (d, J=1.5 Hz, 2H), 4.74 (d, J = 13.9 Hz, 1H), 3.85 (dd, J = 10.25, 4.76 Hz, 1H), 3.65 (m, 1H), 3.56 (m, 4H), 3.15 (m, 2H), 2.96 (m, 3H), 2.07 (m, 2H), 1.37 (m, 2H), 1.22 (m, 2H), 0.84 (t, J = 7.0, 3H).

Protease inhibition assays

The ability of cyclic ureas to inhibit HIV-1 protease was assessed using peptide and polyprotein substrates [16]. K_i values were measured via a fluorescent peptide substrate: Aminobenzoyl-Ala-Thr-His-Gln-Val-*Tyr-Phe(NO$_2$)*-Val-Arg-Lys-Ala, where the scissile bond is indicated by italics. The aminobenzoyl-tagged hexapeptide product was quantified by fluorescence measurement (ex. 330 nm, em. 430 nm) after separation from unreacted substrate by HPLC. K_i values were determined under conditions of substrate and inhibitor excess relative to enzyme concentration using the Michaelis–Menten equation for competitive inhibitors. An *in vitro* transcription/translation product corresponding to all of the HIV-1 gag p17 protein plus the first 78 amino acids of HIV-1 gag p24 and including a single cleavage site for the protease (Val-Ser-Gln-Asn-*Tyr-Pro*-Ile-Val-Gln-Asn) was used to measure the ability of compounds to inhibit the cleavage of viral polyprotein substrates. This polyprotein cleavage assay is carried out under pH and salt conditions thought to mimic those in infected cells (phosphate buffered saline, pH 6.5). The concentration of compound required to inhibit cleavage of the substrate by 50% was designated the IC_{50}.

The potential for inhibition of the cellular aspartyl proteases renin, pepsin and cathepsin D as well as two chymotrypsin-like enzymes, chymotrypsin and cathepsin G, was examined for DMP 850 and DMP 851. Prior to determining specificity of inhibition, the stability of compounds at the various pH values utilized for these assays was assessed using HPLC. Both cyclic ureas were stable under all pH conditions tested, and in human plasma. Some decrease in compound recovered occurred in rat plasma after 4 h, but no metabolites could be identified. Compounds were next examined for their ability to inhibit various enzymes. The aspartic acid protease renin was assayed using a peptide substrate and HPLC detection of substrate remaining and product formed. Cathepsin D and pepsin were assayed for their ability to cleave the protein substrate hemoglobin at acidic pH by measurement of acid-soluble cleavage products via absorbance at 280 nm. The effect of compounds on the chymotryptic-like enzymes chymotrypsin and cathepsin G was assayed using a synthetic peptide substrate and detection of derivatized product by continuous spectrophotometric assay. The test concentrations of compounds were selected to reflect levels at least 250 times that required to inhibit HIV protease by 50% (IC_{50}) as determined against the viral polyprotein GAG substrate. and greater than 250,000 times the apparent K_i value for the viral protease.

Antiviral assays

The ability of DMP 850 and DMP 851 to inhibit HIV replication in tissue culture was assessed using four different assay systems as utilized previously for DMP 450 [8]. The yield of infectious virus produced in acute infections of MT-2 cells was measured using a highly sensitive plaque assay. The concentration of inhibitor required to reduce the yield of infectious virus by 90% (IC_{90}) was determined. The antiviral activity of DMP 850 and DMP 851 was also determined by measurement of viral RNA accumulation in HIV-1(RF) acutely infected MT-2 cells. The concentration of inhibitor that decreased by 90% the level of viral RNA relative to untreated, infected controls was designated the IC_{90}. The effect of cyclic ureas on production of viral p24 antigen by HIV-infected PBMCs or MT-2 cells was measured using the AIDS Clinical Trials Group/Department of Defense PBMC consensus assay (for PBMCs) or by the method of Vacca *et al.* [27] for MT-2 cells. The effect of cyclic ureas on production of viral p24 was also measured in acutely infected MT-4 cells by the method of Vacca *et al.* [27]. The DuPont p24 ELISA kit was used for all p24 quantitation. The concentration of inhibitor required to reduce p24 antigen production by 90% (IC_{90}) was determined.

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