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Potency and Selectivity of Inhibition of Human Immunodeficiency Virus Protease by a Small Nonpeptide Cyclic Urea, DMP 323

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DMP 323 is a potent inhibitor of the protease of human immunodeficiency virus (HIV), with antiviral activity against both HIV type 1 and HIV type 2. This compound is representative of a class of small, novel, nonpeptide cyclic urea inhibitors of HIV protease that were designed on the basis of three-dimensional structural information and three-dimensional database searching. We report here studies of the kinetics of DMP 323 inhibition of the cleavage of peptide and HIV-1 *gag* polyprotein substrates. DMP 323 acts as a rapidly binding, competitive inhibitor of HIV protease. DMP 323 is as potent against both peptide and viral polyprotein substrates as A-80987, Q8024, and Ro-31-8959, which are among the most potent inhibitors of HIV protease described in the literature to date. Incubation with human plasma or serum did not decrease the effective potency of DMP 323 for HIV protease, suggesting that plasma protein binding is of a low affinity relative to that of HIV protease. DMP 323 was also assessed for its ability to inhibit the mammalian proteases renin, pepsin, cathepsin D, cathepsin G, and chymotrypsin. No inhibition of greater than 12% was observed for any of these enzymes at concentrations of DMP 323 that were 350 to 40,000 times higher than that required to inhibit the viral protease 50%.

The protease of human immunodeficiency virus (HIV) is responsible for the specific cleavage of two viral polyproteins (31), leading to the production of a set of structural proteins and enzymes essential for the replication of HIV (19, 28). HIV protease is a member of the aspartic acid family of proteases (27). A variety of peptide-based inhibitors of HIV protease have been synthesized and characterized (7, 9, 23, 36). Although many of these compounds are potent inhibitors of HIV protease and of viral replication in vitro, most have limited oral bioavailability (16, 24). DMP 323 (XM323) is representative of a class of novel, orally bioavailable, nonpeptide inhibitors of HIV protease that are designated cyclic ureas (22). DMP 323 has been shown to be a potent inhibitor of HIV type 1 (HIV-1) and HIV type 2 replication; the mean concentration required to inhibit virus replication 90%, as measured via yield reduction, is 0.12 μM (26). The cyclic ureas were designed (22) by use of X-ray crystallographic data from several HIV protease-inhibitor complexes and structure-based design methods and incorporate a molecule mimicking the structural water molecule found in X-ray crystal structures of HIV protease-inhibitor complexes. Through the incorporation of the functional equivalent of the structural water molecule into the preorganized structure of the cyclic diol, low-molecular-weight inhibitors that allowed for a hydrogen bonding network connecting HIV protease flaps with active-site aspartates resulted. We wished to compare these relatively small inhibitors, which provide side-chain functionalities that fill the S2, S1, S1′, and S2′ subsites of the enzyme, with some of the most potent inhibitors described in the literature to date. These peptide-like inhibitors include the C_2-symmetrical diol Q8024 (12, 13), the hydroxypropylene isostere A-80987 (16, 17), and the hydroxyethylimine isostere Ro-31-8959. (32). These inhibitors comprise residues capable of occupying enzyme subsites from S3 to S3′, S3 to S2′, and S3 to S2′, respectively. We report here studies of the kinetics of inhibition of protease HIV by DMP 323 and these other inhibitors, the effect of plasma or serum binding of DMP 323 on its ability to inhibit the protease, and the extent of inhibition by DMP 323 of other aspartic acid proteases and chymotrypsin-like proteases.

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MATERIALS AND METHODS

All inhibitors were synthesized at The DuPont Merck Pharmaceutical Co. Stock solutions of inhibitors were prepared with dimethyl sulfoxide (DMSO) at concentrations of 1 to 5 mg/ml. All dilutions were prepared with DMSO, except as noted below for the examination of the influence of human plasma or serum on the inhibition of HIV protease. Control reaction mixtures for all assays contained equivalent final concentrations of DMSO (less than 10%).

Preparations of HIV protease. Two different forms of recombinant HIV protease were used: a wild-type homodimer and a single-chain tethered dimer incapable of dissociating at low concentrations. Both recombinant enzymes were isolated from inclusion bodies of *Escherichia coli* BL21(DE3) harboring a plasmid containing a synthetic gene encoding the protein sequence of protease from strain HXB2 as previously described (4) and were partially purified by cation-exchange chromatography. The concentration of HIV protease present in the partially purified inclusion bodies was determined by a comparison of the total protein concentration and the relative intensity of the Coomassie blue-stained band corresponding to the 10-kDa monomer (from sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis) (21).

Assays for HIV protease. The ability of DMP 323 to inhibit HIV-1 protease was assessed with peptide and polyprotein substrates in three different assays.

(i) Assay system A. Assay system A utilized a radiolabeled in vitro transcription-translation product, designated *Pst*I *gag* (10), as a substrate to measure the ability of DMP 323 to inhibit the cleavage of viral polyprotein substrates. This substrate includes a single cleavage site (boldface type) for HIV-1 protease (Val-Ser-Gln-Asn-**Tyr-Pro**-Ile-Val-Gln-Asn). The concentration of DMP 323 that inhibited substrate cleavage by 50% was designated the IC_{50}.

To estimate the kinetic constant for inhibition (K_i), peptide substrates were used. Efficient peptide cleavage by HIV-1 protease requires both a lower pH than is optimal for polyprotein substrates and the presence of high concentrations of NaCl (37). Two different assays were used. A continuous spectrophotometric method (assay B) allowed observation of the time of onset of inhibition. A discontinuous method (assay C) based on high-pressure liquid chromatographic (HPLC) measurement of product formation was more sensitive and allowed measurement of the potency of compounds with low K_is.

(ii) Assay system B. For assay system B, the synthetic peptide Ala-Thr-His-Gln-Val-**Tyr-Phe(NO_2)**-Val-Arg-Lys-Ala was used in a continuous spectrophotometric assay in which the rate of decrease in the A_{300} is proportional to the rate of cleavage of the chromogenic peptide (5). The assay was performed by incubating DMP 323, the peptide substrate, and recombinant HIV-1 protease at room temperature in a buffer containing 1.0 M NaCl, 50 mM morpholineethanesulfonic acid (pH 5.5), 1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. The wild-type protease used was the same as that used for assay A.

(iii) Assay system C. For assay system C, a 2-aminobenzoyl group was added to the amino terminus of the peptide substrate described above to create a fluorescent cationic peptide substrate (5). Cleavage yielded a fluorescent anionic product which could be rapidly measured by ion-exchange HPLC. The final enzyme concentration used (0.0625 to 2.5 nM) was much lower than that used in assay A or B. Because of the possibility of dissociation of the enzyme at this concentration, both the wild-type enzyme and a single-chain tethered dimer prepared from *E. coli* inclusion bodies (5) were used. The use of a nondissociable single-chain enzyme allowed the determination of apparent K_is of as low as 0.05 nM. DMP 323 was preincubated for 15 min at room temperature with enzyme (final concentration, 0.0625 to 2.5 nM) in an assay buffer (pH 5.5) containing 1 M NaCl. Substrate was then added (final concentration, 5 to 100 μM), and incubation was continued at 20 to 37°C for 15 min to 1 h. After the reaction was stopped with 0.1 M ammonium hydroxide, the fluorescent product (2-aminobenzoyl-Ala-Thr-His-Gln-Val-Tyr) was separated from the fluorescent substrate by anion-exchange HPLC on a Mono-Q anion-exchange column (Pharmacia), and fluorescence was monitored at an excitation wavelength of 330 nm and an emission wavelength of 430 nm (Shimadzu RF535 detector). The mobile phase used involved a gradient from 100% buffer A at 0 min after injection to 70% buffer A at 5 min after injection, a 5-min wash with 100% buffer B, and a 3-min requilibration with buffer A. Buffer A was 20 mM Tris-HCl (pH 9.0)–0.02% sodium azide–10% acetonitrile, and buffer B was 20 mM Tris-HCl (pH 9.0)–0.02% sodium azide–10% acetonitrile–0.5 M ammonium formate. The flow rate was 1.0 ml/min. The retention time for the product was 3.6 min. K_is for inhibitor binding were estimated at fixed concentrations of substrate and inhibitor from fractional activity measurements by use of the rearranged Michaelis-Menten equation: $K_i = I/(\{[K_m + S - (fa \times S)]/(fa \times K_m)\} - 1)$, where I = inhibitor concentration, S = substrate concentration, fa = fractional activity (or percent activity), and K_m = Michaelis constant. The K_is reported are averages of two or three values obtained with concentrations of inhibitor and substrate and incubation times giving fractional activities of 0.1 to 0.8 relative to those in uninhibited control incubations.

Measurement of enzyme inhibition in the presence or absence of human serum or plasma. The effect of human plasma or serum on the inhibitory potency of DMP 323 was examined. Stock solutions (2.5 mM) of DMP 323 in DMSO were added to plasma, serum, or phosphate-buffered saline (PBS; 20 mM sodium phosphate [pH 7.0], 0.15 M NaCl) at a final concentration of 67 μg/ml (118 μM) and fivefold serially diluted in plasma, serum, or PBS to a lowest concentration of 0.021 μg/ml (37 nM). Control samples contained no DMP 323 but contained plasma, serum, or PBS. Aliquots (1 μl) of these mixtures were then added to HIV protease and a chromogenic peptide substrate in a pH 5.5 buffer containing 1.0 M NaCl, and the decrease in the A_{300} was monitored for 10 min. The slope corresponding to the rate of chromogenic peptide cleavage was used to calculate percent inhibition relative to that in the spectrophotometric reaction containing only substrate, enzyme, and buffer. The amount of endogenous inhibition in the serum, plasma, and PBS control samples lacking DMP 323 was subtracted from that in the corresponding test samples. The net percent inhibition versus the amount of DMP 323 was plotted, and the concentration of DMP 323 yielding 50% inhibition was compared for the three conditions.

Assays for other proteases. The aspartic acid protease renin was assayed as described by Poe et al. (30) by use of a tetradecapeptide fragment (DRVYIHPFHLLVYS; Calbiochem) of the natural renin substrate angiotensinogen and reverse-phase HPLC to separate substrate and product peptides. The A_{220} was monitored. A synthetic peptide inhibitor of renin, PHPFHFFVYK, inhibited renin-catalyzed substrate cleavage 88% at 1.3 μM. This synthetic peptide has been reported to be a specific inhibitor of human renin in vivo (3, 6).

The aspartic acid protease pepsin was assayed by use of acid-denatured hemoglobin (11). DMP 323 was incubated at 37°C at final concentrations of 42 μM to 1.92 mM with human hemoglobin (82 mg/ml; Sigma) in 60 mM HCl and porcine pepsin (2 μg/ml). After 30 min, protein was precipitated by the addition of trichloroacetic acid, and the A_{280} of the supernatant was measured. Pepstatin A at 50 nM inhibited pepsin-catalyzed hemoglobin cleavage 75%; the reported IC_{50} for pepstatin A inhibition of pepsin is 14 nM (15). Inhibition of the aspartic acid protease cathepsin D was assayed as described by Takahashi and Tang (35).

The serine proteases cathepsin G and chymotrypsin were assayed at room temperature by a continuous spectrophotometric assay with a synthetic peptide substrate, succinyl-Ala-Ala-Pro-Phe-nitroanilide (1, 8, 25). Concentrations of enzymes and substrate were chosen to achieve linear rates of peptide hydrolysis over a 10-min reaction period.

RESULTS

Effects of DMP 323 on polyprotein and peptide cleavage by HIV protease. A large number of peptidolytic and reporter protein-based assays have been described for HIV protease (reviewed in reference 2). Viral polyprotein-based assays provide a measure of potency under conditions of salt concentration and pH mimicking those in the cellular environment, while peptide-based assays are more facile, sensitive, and quantitative. We determined the potency of DMP 323 against HIV protease by using both viral polyprotein and synthetic

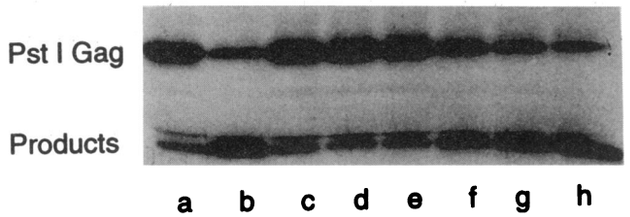

FIG. 1. Effect of DMP 323 on the cleavage of HIV *gag* polyprotein by HIV protease. An in vitro translation product corresponding to all of p17 and a portion of p24 was incubated together with 5 μg of HIV protease per ml and various concentrations of DMP 323 in PBS (pH 6.5) for 30 min at 30°C. Products and the substrate were separated on SDS–8 to 16% polyacrylamide gels and processed for fluorography with Amplify (Amersham). A photograph of the fluorogram is shown. Lanes: a, sample incubated without HIV protease or DMP 323; b, sample incubated without the inhibitor; c to h, samples incubated with DMP 323 at 12.5 μM, 1.25 μM, 125 nM, 25 nM, 5 nM, and 1 nM, respectively.

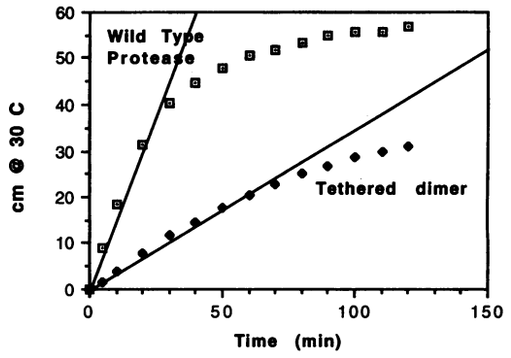

FIG. 3. Linearity of protease activity with time. Wild-type HIV PR and tethered dimeric HIV protease (0.25 nM) were incubated at 30°C with 5 μM fluorescent peptide substrate for various times. The amount of product formed during each incubation was measured by HPLC (assay C) and is shown as cm peak height.

peptide substrates under conditions optimal for the substrates examined.

DMP 323 inhibited the cleavage of viral polyprotein substrates, as shown in Fig. 1. On the basis of the quantitation of substrate disappearance, the IC_{50}s were 31.6 nM and 39.8 nM in two separate measurements.

DMP 323 inhibition of peptide cleavage by wild-type HIV protease was nearly instantaneous (Fig. 2, assay B). This behavior contrasts with that of many tightly binding inhibitors, or reaction intermediate analogs, which exhibit slow binding, with half-lives of association of minutes to hours (33). The increase in the level of inhibition with the addition of 5.3 to 21.3 nM DMP 323 is linear with respect to inhibitor concentration. Such titration behavior allows extrapolation to an active-site concentration (5) of ~30 nM for these reaction mixtures (containing 120 nM potential HIV protease dimers, as determined by SDS-polyacrylamide gel staining) and suggests that the K_i is much lower than the concentration of the inhibitor used. To permit the determination of K_is that are subnanomolar, it is necessary to use a highly sensitive assay, such that lower concentrations of both the enzyme and the inhibitor can be used, while keeping the HIV protease concentration much lower than the inhibitor concentration. At the same time, the dissociation of HIV protease dimers occurs in a concentration- and pH-dependent manner (5). The reported dissociation constant, K_d, at pH 7.0 is 50 nM when measured with the fluorescent substrate used here (5). Both higher and lower K_ds have been reported with other conditions of salt and organic cosolvent (14, 20). The use of tethered dimeric HIV protease precludes a dissociation-mediated time-dependent loss of enzyme activity and allows enzyme concentrations of as low as 0.0625 nM to be used.

Potency of DMP 323 for the tethered dimer versus the wild-type protease. At pH 5.5 and 25 to 37°C, the protease activity of the tethered dimer was linear with time for more than 60 min and linear with protein concentration from 0.0625 to 2.5 nM (assay C). The duration of linearity with the wild-type enzyme was considerably shorter than that with the tethered dimer (Fig. 3), most likely because of dissociation of the wild-type protease homodimer. As expected from the dissociation considerations noted above, the linearity of HIV protease activity increased with enzyme concentration and decreased with temperature between 20 and 37°C. When assessed under conditions in which protease activity was linear

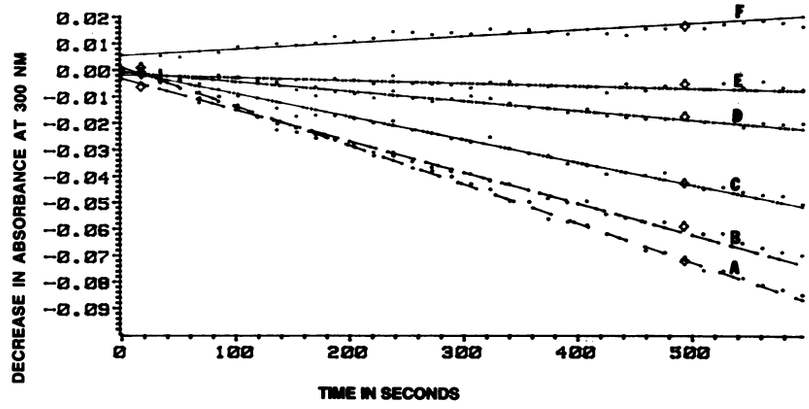

FIG. 2. Time-dependent decrease in the absorbance of the chromogenic peptide substrate due to cleavage by wild-type HIV protease. The substrate and enzyme concentrations were 0.225 mM and 2.5 μg/ml, respectively. The temperature was 25°C. The concentrations of DMP 323 in reactions corresponding to lines A to E were 0, 5.3, 10.6, 21.3, and 42.6 nM, respectively. Line F corresponds to reactions containing the substrate but lacking HIV protease. Dots represent measurements taken at 15-s intervals. Diamonds show the period for which a best-fit straight line was calculated.

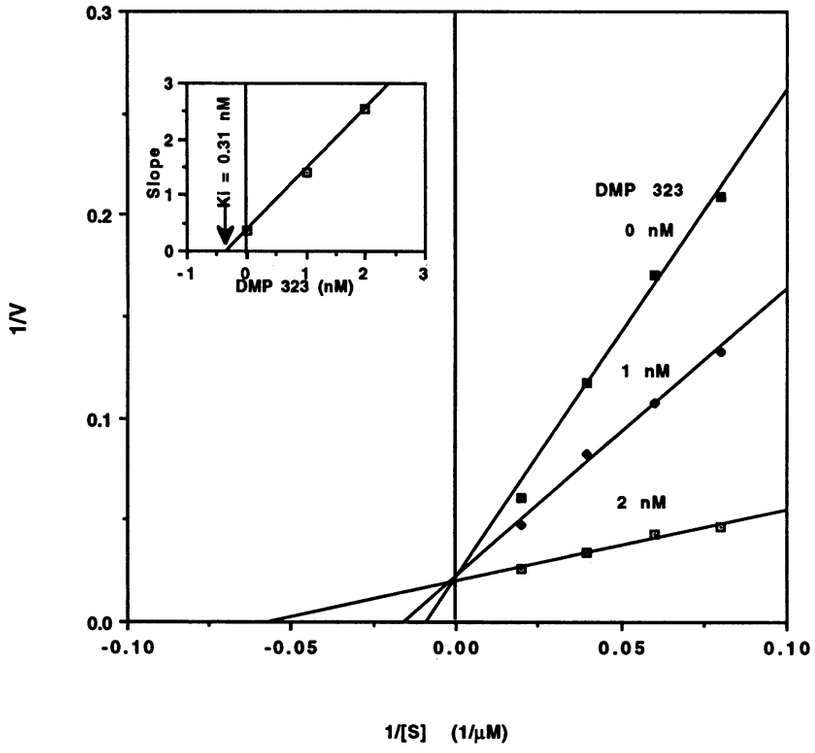

FIG. 4. Lineweaver-Burk plot of the inhibition of HIV protease by DMP 323. Tethered dimeric HIV protease (0.25 nM) was incubated with 12.5 to 50 μM peptide substrate (S) and 0, 1, or 2 nM DMP 323. The formation of cleavage products (V) was measured by assay C.

for 1 h (0.0625 to 0.25 nM tethered dimer measured at 30°C; 0.25 nM wild-type enzyme measured at 20°C), the two enzymes behaved in a functionally equivalent manner, as previously described (5). The kinetic constants for the tethered dimer and the wild-type enzyme, respectively, were as follows: K_m, 18 and 30 μM; V_{max}, 2,600 and 7,100 nmol of product per nmol of enzyme per h; and k_{cat} (catalytic constant), 0.72 and 1.9 s^{-1}. The K_i for DMP 323 obtained with the tethered dimeric form of HIV protease was 0.27 ± 0.12 nM (n = 13).

To examine the nature of inhibition by DMP 323, activity was measured in duplicate at a fixed enzyme concentration of 0.25 nM, substrate concentrations from 12.5 to 50 μM, and a DMP 323 concentration of 0, 1, or 2 nM. The y intercepts in a Lineweaver-Burk plot were not significantly different, suggesting that inhibition was of the competitive type (Fig. 4). A K_i value can also be obtained from a replot of the slopes of the data from Fig. 4. This K_i value is therefore derived from all 24 datum points rather than, as in the routine assay that we perform on new compounds, two or three inhibitor concentrations and one substrate concentration. The K_i value obtained from the intercept, as shown in the inset in Fig. 4 (0.31 nM), is similar to that obtained (0.27 ± 0.12 nM) by calculating K_i values from two or three single points on each of 13 days.

We compared the potency of DMP 323 with those of other protease inhibitors in these assays (Table 1). The potency of DMP 323 in assays of both peptide cleavage and polyprotein cleavage is comparable to those of HIV protease inhibitors currently being pursued as potential therapeutic agents for AIDS.

Effect of human plasma or serum on the potency of DMP 323. Many drug substances bind extensively to plasma proteins, thus limiting the effective concentration available for binding to target molecules. Plasma protein binding of DMP 323 is estimated to be 80% or more, depending on the plasma species (34). The effect of human plasma or serum on the concentration-dependent inhibition of HIV protease was examined. The ability of DMP 323 to inhibit HIV protease in the presence or absence of human serum or plasma was examined by comparing DMP 323 concentration-percent inhibition curves by use of assay B. High-affinity and/or irreversible binding by proteins or other components present in human plasma or serum would be detected by a decrease in the level of enzyme inhibition observed in the assay, manifested by a shift to the right of the DMP 323 concentration-percent inhibition curves. The curves for the three conditions are shown in Fig. 5. The concentrations of DMP 323 plotted on the x axis correspond to the final concentrations of DMP 323 used in assay B, as described in Materials and Methods.

There was an average 20% inhibition of enzyme activity

TABLE 1. Comparative potencies of DMP 323 and other HIV protease inhibitors[a]

Inhibitor	IC_{50} (nM) in assay A	K_i (nM) in assay C for:	
		Tethered dimer	Wild-type enzyme
DMP 323	35.7 ± 5.8	0.27 ± 0.12	0.94 ± 0.39
Q8024	45.6 ± 20.4	0.22 ± 0.08	Not determined
Ro-31-8959	13.1 ± 5.2	0.25 ± 0.1	0.14 ± 0.04
A-80987	125 (n = 1)	0.54 ± 0.16	0.61 ± 0.18

[a] Values are means ± standard deviations for 3 to 13 separate assays, unless noted otherwise.

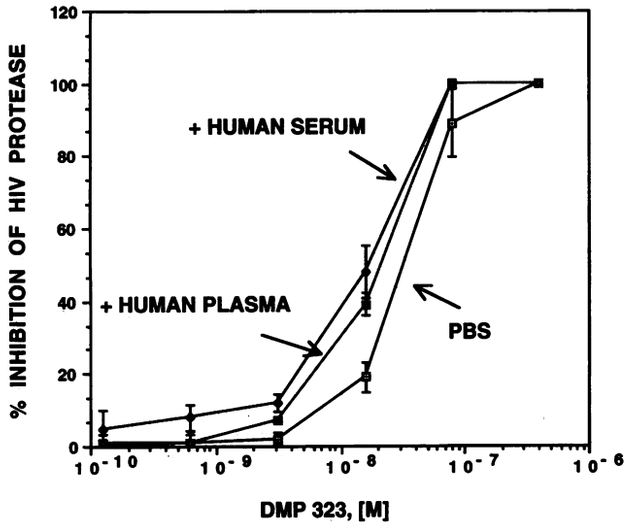

FIG. 5. Effect of preincubation with human plasma or serum on the inhibitory potency of DMP 323. Serial dilutions of DMP 323 were prepared in human plasma, serum, or PBS as described in the text, and the inhibition of HIV protease was measured by use of a continuous spectrophotometric assay (assay B). The DMP 323 concentrations plotted correspond to those present in the spectrophotometric reactions.

because of the presence of plasma or serum alone; the data were first corrected for this background inhibition. As shown in Fig. 5, there was no decrease in DMP 323 activity as a result of incubation with human serum or plasma. The apparent IC_{50}s were 9.3, 11.7, and 15.6 nM for serum, plasma, and PBS incubations, respectively. The results suggest that there may even be some enhancement of DMP 323 activity in the presence of human serum or plasma. These results indicate that binding to plasma or serum components does not affect the ability of DMP 323 to inhibit HIV protease.

Selectivity of DMP 323 for the inhibition of HIV protease. The potential for DMP 323 inhibition of mammalian proteases with overlapping or similar substrate specificities was examined. These included other aspartic acid proteases (renin, pepsin, and cathepsin D) and chymotrypsin-like proteases, which prefer hydrophobic amino acids, such as phenylalanine, at the P1 or P1′ position. Test concentrations of DMP 323 were selected to reflect levels greater than 350 times the level required for 50% inhibition of HIV protease when assayed with a viral polyprotein substrate and over 40,000 times the level required for 50% inhibition of the protease with peptide substrates. Pepsin inhibition was assessed at a peak DMP 323 concentration of 1.9 mM, which corresponds roughly to the concentration achievable in the stomach after fasting and following a single dose of 5 mg/kg of body weight. For all the enzymes examined, no more than 12% inhibition of enzyme activity was observed, indicating that DMP 323 shows a high degree of specificity for HIV protease. The highest test concentrations studied and the resultant inhibition by DMP 323 of the various enzymes are shown in Fig. 6.

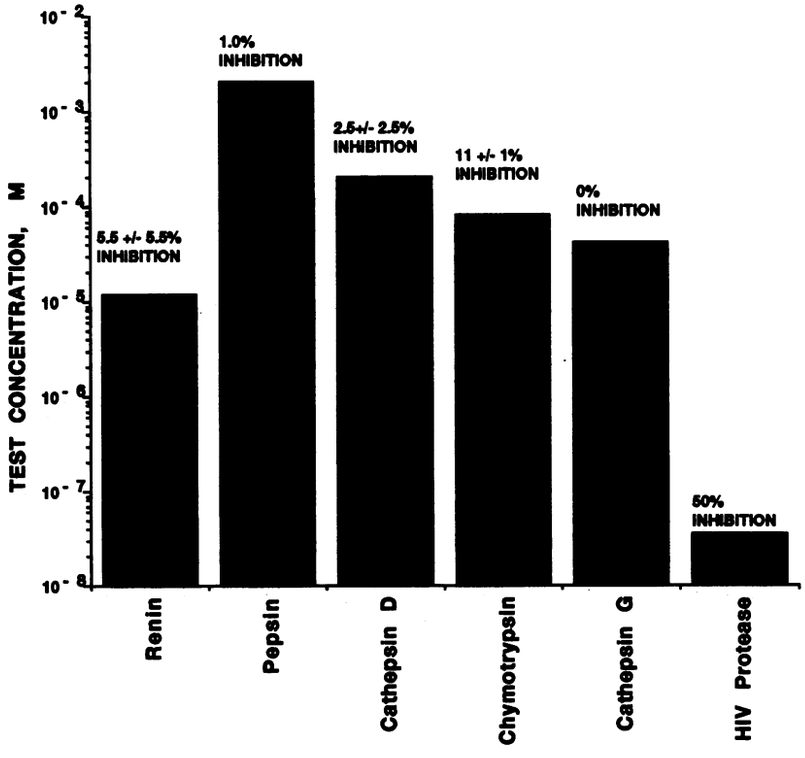

FIG. 6. Selectivity of DMP 323 for HIV protease. Inhibition of various aspartic acid and chymotrypsin-like proteases was determined as described in the text. The highest concentrations of DMP 323 used in the various assays are indicated by the height of the bars. The actual percent inhibition measured is indicated at the top of the bars. Values represent means ± standard deviations for three to five determinations.

DISCUSSION

A variety of transition state mimetic inhibitors of HIV protease have been described, with potencies in the nanomolar or subnanomolar range (7, 9, 16, 23, 32, 36). However, these potent inhibitors are pseudopeptides (containing one or more amide bonds) or molecules containing substrate-like linear scaffolds. Despite extensive structure-activity studies, it has been difficult to combine adequate potency with significant oral bioavailability (16, 24). The difficulty in turning peptide leads into useful HIV therapeutic agents is, of course, not unique to HIV protease inhibitors; extensive research in the area of renin inhibitors has been aimed at meeting this challenge (18). Peptide-based molecules are, in general, of an undersirably large size (>600 Da), poorly absorbed, and rapidly metabolized (29). Cyclic ureas, such as DMP 323, represent a new approach to the inhibition of HIV protease. They are not peptide-like inhibitors but rather are examples of a novel class of compounds which were designed to combine high potency with small size.

To determine the relative potencies of DMP 323 (molecular weight, 567) and larger, potent inhibitors (molecular weights, 654 to 767) described in the literature, we used a series of assays that would allow us to determine inhibitory potency. We used both natural (polyprotein) substrates under conditions mimicking those in cells and peptide substrates, which allowed highly sensitive quantitative assays. DMP 323 was as potent against both peptide substrates and viral polyprotein substrates as protease inhibitors that have been described in the literature to date, despite the fact that DMP 323 occupies only four of the eight available enzyme subsites, while the other compounds occupy five or six of the eight sites. As previously described (22), the potency of the cyclic ureas is a result of several factors, including displacement of the water molecule by the cyclic urea carbonyl oxygen, preorganization of the P1, P1′, P2, and P2′ substituents for highly complementary binding to the enzyme subsites, and hydrophobic and hydrogen bonding interactions between the P2 and P2′ substituents and the S2 and S2′ enzyme pockets. The resulting relatively small size of the cyclic ureas (<600 Da) contributes to the oral bioavailability in animals that has been observed for members of this series (22, 38).

DMP 323 showed a high degree of specificity for HIV protease in comparison with other mammalian aspartic acid proteases and chymotrypsin-like enzymes. This exquisite specificity for HIV protease was not unexpected, since only in the retroviral proteases has the structural water molecule that the cyclic urea framework mimics been shown to play a role in inhibitor binding.

Incubation with human plasma or serum did not decrease the effective potency of DMP 323 against HIV protease. However, DMP 323 has been found to bind to plasma proteins fairly extensively; binding to human plasma is 88.4% (34). The fact that this plasma protein binding does not translate to a diminished ability to inhibit HIV protease suggests that DMP 323 binding to plasma or serum proteins is of a relatively low affinity, such that dilution in the assay system containing the target HIV protease results in dissociation from the potential binding sites.

In summary, DMP 323 has high potency and selectivity for HIV protease. The characteristics of potency and selectivity described here suggest that as a class, the cyclic ureas are compounds suitable for further development as AIDS therapeutic agents. Unfortunately, despite substantial oral bioavailability in animals (38), variable absorption of DMP 323 in humans was observed, due in part to the limited aqueous solubility of DMP 323. Cyclic ureas with improved pharmacokinetic properties are currently under investigation.

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