Substrates and Inhibitors of Human T-cell Leukemia Virus Type I Protease

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ABSTRACT: HTLV-I is an oncogenic retrovirus that is associated with adult T-cell leukemia. HTLV-I protease and HTLV-I protease fused to a deca-histidine containing leader peptide (His-protease) have been cloned, expressed, and purified. The refolded proteases were active and exhibited nearly identical enzymatic activities. To begin to characterize the specificity of HTLV-I, we measured protease cleavage of peptide substrates and inhibition by protease inhibitors. HTLV-I protease cleavage of a peptide representing the HTLV-I retroviral processing site P19/24 (APQVLPVMHPHG) yielded K_m and k_{cat} values of 470 μM and 0.184 s^{-1} while cleavage of a peptide representing the processing site P24/15 (KTKVLVVQPK) yielded K_m and k_{cat} values of 310 μM and 0.0060 s^{-1}. When the P1′ proline of P19/24 was replaced with *p*-nitro-phenylalanine (Nph), the ability of HTLV-I protease to cleave the substrate (APQVLNphVMHPL) was improved. Inhibition of HTLV-I protease and His-protease by a series of protease inhibitors was also tested. It was found that the K_i values for inhibition of HTLV-I protease and His-protease by a series of pepsin inhibitors ranged from 7 nM to 10 μM, while the K_i values of a series of HIV-1 protease inhibitors ranged from 6 nM to 127 μM. In comparison, the K_i values for inhibition of pepsin by the pepsin inhibitors ranged from 0.72 to 19.2 nM, and the K_i values for inhibition of HIV-1 protease by the HIV protease inhibitors ranged from 0.24 nM to 1.0 μM. The data suggested that the substrate binding site of HTLV-I protease is different from the substrate binding sites of pepsin and HIV-1 protease, and that currently employed HIV-1 protease inhibitors would not be effective for the treatment of HTLV-I infections.

Human T-cell leukemia virus type I is an oncovirus in the *Retroviridae* family (*1*). HTLV-I[1] was first isolated in the early 1980s from patients with adult T-cell leukemia/lymphoma (*2*), and has been shown to be clinically associated with adult T-cell leukemia/lymphoma (*3*), HTLV-I associated myelopathy/tropical spastic paraparesis (TSP) (*4*), and a number of other chronic diseases (i.e., uveitis, arthritis, and infective dermatitis) (*5*, *6*).

The genome of HTLV-I is approximately 9 kb in length and is flanked by long terminal repeats. Like other human retroviruses there are three large open reading frames that encode Gag (48 kDa), Pol (99 kDa), and Env (54 kDa) proteins, and a number of spliced, open reading frames that encode regulatory proteins (Rex, Tax, etc.) (*7*, *8*). Proteolytic cleavage of HTLV-I Gag protein yields matrix, capsid, and nucleocapsid proteins of the mature virion, while proteolytic cleavage of the Pol protein presumably produces HTLV-I reverse transcriptase and integrase. The protease responsible for processing of the Gag and Gag-Pol proteins is virally encoded, and the gene (*pro*) is found between the genes for Gag (*gag*) and Pol (*pol*). (Figure 1)

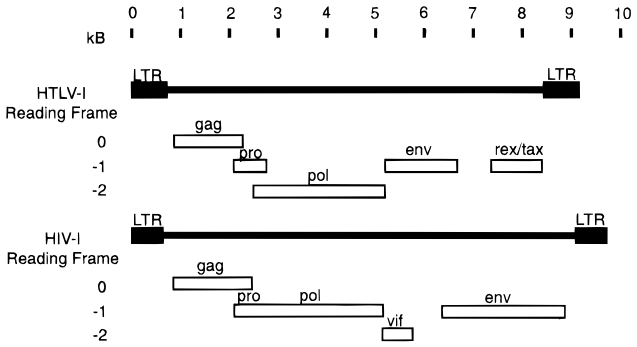

FIGURE 1: A schematic representation of the major genes of HTLV-I and HIV-1. A length scale in kb is shown at the top of the diagram. "Reading Frame" refers to the translation frame of the illustrated genes relative to *gag* (*gag* was arbitrarily assigned as the 0 reading frame). Note that many of the small genes and/or spliced genes of both viruses have been omitted to improve the clarity of the figure.

The organization of HTLV-I *gag*, *pro*, and *pol* is typical of type C retroviruses and is different from the organization of *gag*, *pro*, and *pol* in the more familiar type D retrovirus HIV (*9*−*11*). In HIV, the reading frame of *pol* is shifted in the −1 direction from *gag*, and *pro* is actually part of the 5′ end of *pol*, while in HTLV-I, *gag*, *pro*, and *pol* are all separate genes that are encoded in different reading frames.

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[1] Abbreviations: Amp, ampicillin; CH_3CN, acetonitrile; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; His-protease or His-PR, HTLV-I protease fused to the his-tag leader of pET19b; HPLC, high-performance liquid chromatography; HTLV-I, Human T-cell leukemia virus type I; IPTG, isopropyl-β−D-thiogalactopyranoside; kb, kilo base pairs; LB, Luria-Bertani medium; Nph, *p*-nitro phenylalanine; PR, HTLV-I protease.

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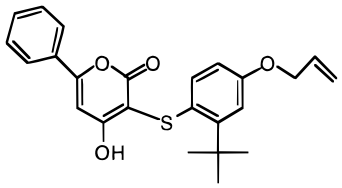

FIGURE 2: Structure of MES13-099.

The reading frame of HTLV-I *pro* is shifted in the −1 direction from *gag*, and the reading frame of *pol* is shifted in the −1 direction from *pro*. As a consequence, two ribosomal frame shifts, one in the *gag-pro* overlap and one in the *pro-pol* overlap, are required to synthesize the *pol*-encoded replication enzymes of HTLV-I.

HTLV-I protease is an aspartic acid protease like HIV protease, RSV protease, cathepsin D, and pepsin, and has been expressed in *Escherichia coli* (12−15). The processing sites of a few of the HTLV-I precursor proteins have been identified (*10*, *12*, *16*, *17*), but little else is known about the protease.

Within the past 5 years HIV protease inhibitors have become one of the primary drugs for the treatment of HIV infections (*18*−*20*). Unfortunately, analogous protease inhibitors for the treatment of HTLV-I infections and the prevention of HTLV-I-induced leukemia are unknown. We have previously reported an efficient method for expression and purification of HTLV-I protease (*21*). To begin to characterize the specificity of HTLV-I protease, we report here quantitative measurements of the cleavage of peptides representing HTLV-I protease sites and the inhibition of HTLV-I protease by aspartic acid protease inhibitors.

EXPERIMENTAL PROCEDURES

Substrates. Synthetic peptides APQVLPVMHPHG (P19/24), KTKVLVVQPK (p24/15), and APQVLNphVMHPL were purchased from SynPep Corp.

Inhibitors. HIV-1 protease inhibitors N1270, N1395, N1460, and N1465 were purchased from BACHEM. The synthesis of the aspartic acid protease inhibitors, MES13-099 (*22*, *23*), CS-I-22 (*24*), CS-I-25 (*24*), CS-I-27 (*24*), CS-I-51 (*24*), CS-I-52 (*24*), and JG-365 (*25*, *26*), has been previously reported, and the structures of the inhibitors are shown in Tables 2 and 3 and Figure 2. MES13-099 (Figure 2, ref *22*) is an analogue of the Parke Davis pyrone HIV protease inhibitors (*23*).

Purification of HTLV-I protease and His-protease. The previously reported purification of HTLV-I protease (*21*) was modified in order to recover both purified HTLV-I protease and His-protease (Figure 3).

Cultures (30 mL) of *E. coli* BL21(DE3)pLysS containing the protease expression plasmid pPR101 (*21*) were grown at 37 °C in LB/Amp[1] to an OD_{600} of 0.6 and induced by addition of IPTG (0.4 mM final concentration). Three hours after the addition of IPTG, the cells were harvested by centrifugation, resuspended in buffer A (20 mM Tris, pH 7.9, 5 mM imidazole, and 500 mM NaCl), and sonicated. The bacterial lysate was cleared by centrifugation, and the pellet was resuspended in Buffer B (Buffer A plus 8 M urea). The mixture was cleared by centrifugation, and the supernatant was then loaded on a 1 mL His-Bind (Novagen) column. The column was then washed with Buffer B and Buffer C (20 mM Tris, pH 7.9, 20 mM imidazole, 500 mM NaCl, and 8 M urea) and eluted with Buffer D (20 mM Tris, pH 7.9, 1 M imidazole, 500 mM NaCl, and 8 M urea) under denaturing conditions. A 20 kDa protein bound to the His-Bind Resin affinity column and eluted with imidazole. A protein of this size corresponds to the expected molecular weight of HTLV-I protease fused to the 27 amino acid pET19-b histidine tag (Figure 3; The fusion protein will be referred to as His-protease).

The purified fusion protein was refolded by sequential dialysis against buffer E (10 mM sodium acetate buffer, pH 3.5) and buffer F (100 mM sodium citrate buffer, pH 5.3, 5 mM EDTA, 1 mM DTT, and 1 M NaCl). Autoprocessing of His-protease occurred during dialysis against buffer F, and the fully processed protein coprecipitated with unprocessed His-protease. Refolded unprocessed His-protease remained in solution and more than 500 μg of purified His-protease was obtained from a 30 mL culture. After 8 h of dialysis, the precipitated protein was collected by centrifugation at 20000*g* for 20 min at 40 °C, redissolved in buffer B, and reloaded on a second His-Bind column. The mature protease was found in the fractions flowing through the His-Bind column, and these fractions were pooled, dialyzed against buffer E containing 1 mM DTT, and stored at 40 °C. Typically 100 μg of HTLV-I protease was obtained from a 30 mL culture.

Chemicals. Citric acid and sodium citrate were purchased from Bio-Rad. EDTA, dithiothreitol (DTT), NaCl, trifluoroacetic acid (TFA), and acetonitrile (CH3CN, HPLC grade) were from Fisher. All chemicals were reagent grade or better.

HPLC System. The HPLC system was a Hewlett-Packard Series II 1090 liquid chromatograph. Filters with wavelengths of 210 and 269 nm were used in the UV detector. The C_{18} reverse-phase column was purchased from Waters (C_{18}, 300 Å, 3.9 × 150 mm).

Protease Activity Assays. The activity of both His-protease and mature HTLV-I protease was determined by measuring the rate of hydrolysis of the synthetic peptides P19/24 (APQVLPVMHPHG) (*14*, *15*) and P24/15 (KTKVLVVQPK) (*13*). P19/24 and P24/15 represent the HTLV-I *gag* protein cleavage sites found at the junctions of the p19, p24, and p15 proteins.

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                                    ↓
        10         20         30         40         50         60
MGHHHHHHHH HHSSGHIDDD DKHMLEDPVI PLDPARRPVI KAQVDTQTSH PKTIEALLDT
        70         80         90        100        110        120
GADMTVLPIA LFSSNTPLKN TSVLGAGGQT QDHFKLTSLP VLIRLPFRTT PIVLTSCLVD
       130        140        150
TKNNWAIIGR DALQQCQGVL YLPEAKGPPV IL
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FIGURE 3: The predicted amino acid sequence of the recombinant HTLV-I protease fusion protein. The arrow indicates the site of autoprocessing and the histidine tag is underlined.

Table 1: Cleavage of Peptide Substrates by HTLV-I Protease and His-protease

substrate[a]	enzyme	K_m (μM)	k_{cat} (s^{-1})
APQVL/PVMHPHG (p19/24)	His-PR	410 ± 210	0.178 ± 0.061
	mature PR	470 ± 130	0.184 ± 0.024
KTKVL/VVQPK (p24/15)	His-PR	240 ± 50	0.0068 ± 0.0016
	mature PR	310 ± 60	0.0060 ± 0.0004
APQVL/NphVMHPL	His-PR	78 ± 18	20.2 ± 4.3
	mature PR	61 ± 18	21.6 ± 4.6

[a] The "/" indicates the cleavage site.

The reactions (100 μL) contained 1−5 μM protease and 30−180 μM substrates (either p19/24 or p24/15) in buffer F (100 mM sodium citrate, 5 mM EDTA, 1 mM DTT, and 1 M NaCl, pH 5.3). Reactions were incubated at 37 °C for 30 min, and the product peptides, either APQVL and PVMHPHG or KTKVL and VVQPK, were separated and measured on a C_{18} HPLC column (*13*). Protease assays using the modified peptide, APQVLNphVMHPL, were as described above, except that the protease concentration was reduced to 1 nM and the incubation time was reduced to 15 min.

HPLC Elution Conditions. The peptides P19/24 and P24/15 and their cleavage products could be separated on a reverse-phase C_{18} column by eluting with a linear gradient of 10−25% acetonitrile (0.1% TFA). The elution conditions for the modified peptide APQVLNphVMHPL and its products were a linear gradient of 30−45% acetonitrile (0.1% TFA).

The elution of the native peptides (P19/24 and P24/15) and the modified peptide was monitored at 210 and 269 nm, respectively.

Inhibition Assays. All of the inhibitors were dissolved in dimethyl sulfoxide (DMSO) to make a 10 mM stock solution.

Inhibitors were preincubated with protease (either mature protease or His-protease) for 5 min at 37 °C prior to the addition of the modified substrate (APQVLNphVMHPL). Protease activity was then determined as described above.

Determination of K_m, V_{max}, and k_{cat} Values. The initial velocity of proteolysis was determined by measuring the disappearance of substrate. The data were analyzed by the methods of Lineweaver and Burke, and Eadie and Hofstee, and the K_m, V_{max}, and k_{cat} values were calculated (*27*).

Calculation of K_i Values. Inhibition constants (K_i) were determined by the initial velocity method of Dixon (*27*).

RESULTS

Protease Activity. The activity of both mature HTLV-I protease and His-protease was determined by measuring the rates of hydrolysis of the synthetic peptides P19/24 (APQVLPVMHPHG) and P24/15 (KTKVLVVQPK). Reactions were incubated at 37 °C for 30 min, and product peptides, either APQVL and PVMHPHG, or KTKVL and VVQPK, were observed by HPLC. This suggested that the purified His-protease and HTLV-I protease were active. It was also observed that the peptide hydrolytic activity of the purified proteases obeyed simple Michaelis−Menten kinetics and that K_m and k_{cat} values could be determined (Table 1).

In studies of HIV protease, it was reported that peptide substrates containing *p*-nitro-phenylalanine at the C-terminal position of the peptide bond being hydrolyzed (the P1′ position (*28*)) were more easily cleaved by the protease (*29*, *30*). An analogous substrate has also been tested with HTLV-I protease (*12*). This modified HTLV-I substrate is APQVLNphVMHPL. In this substrate the amino acid residue at the P1′ site in P19/24 is replaced by *p*-nitro-phenylalanine (Nph).

The ability of HTLV-I protease to cleave this modified substrate was improved. Table 1 lists the kinetic parameters for cleavage of APQVLNphVMHPL by His-protease and HTLV-I protease. The K_m was reduced, and the k_{cat} was increased by 100.

Comparison of the K_m and k_{cat} values (and the K_i values in the next section) of HTLV-I protease and His-protease revealed that the enzymes are kinetically equivalent. This suggested that His-protease and mature protease are enzymatically similar, and that the leader peptide of His-protease did not radically alter the enzymatic properties of the protease.

Inhibition of HTLV-I Protease and His-protease by Pepsin Inhibitors. Inhibition of HTLV-I protease and His-protease by a series of peptidomimetic pepsin inhibitors was measured to determine the relationship of the peptide binding site of HTLV-I protease to the binding site of pepsin, and to determine whether mature HTLV-I protease and His-protease are comparable.

CS-I-22, CS-I-25, CS-I-27, CS-I-51, and CS-I-52 are a series of peptidomimetic compounds that are porcine pepsin inhibitors (*24*). HTLV-I protease and His-protease are equally inhibited by these pepsin inhibitors and exhibit K_i values that range from 7.2 nM to 10 μM (Table 2). If the K_i values of CS-I-51 for HTLV-I protease and for pepsin are used as standards for comparison, then the data showed that the K_i of CS-I-22 decreased by a factor of 2−3 for HTLV-I protease and a factor of 24 for pepsin. The replacement of the P2 Val and the P2′ Lys in CS-I-51 by Nle and Ala (CS-I-22) improved inhibitor function for both HTLV-I protease and pepsin, but modifications of CS-I-51 did not always produce the same effect for the inhibition of HTLV-I protease and pepsin. When the P3 Val and P2′ Lys of CS-I-51 were replaced by Lys and Ala, the K_i (for CS-I-25) decreased by a factor of 55 for HTLV-I protease but increased by a factor of 25 for pepsin, and when the P2′ Lys and the P3′ Iaa of CS-I-51 were replaced by Ala and Lys-OMe, the K_i (for CS-I-52) increased by a factor of 15 for HTLV-I protease but decreased by a factor of 7 for pepsin. These trends in the K_i data for HTLV-I protease suggested that a P3 Lys improves inhibitor binding while a P3′ Lys interferes with inhibitor binding.

Inhibition of HTLV-I protease by HIV-1 Protease Inhibitors. MES13-099 (Figure 2), JG-365, N-1270, N-1395, N-1460, and N-1465 are inhibitors of HIV-1 protease. Inhibition of HTLV-I protease by these inhibitors was

Table 2: Inhibition of HTLV-I Protease by Pepsin Inhibitors

Inhibitor (molecular mass)	Structure[a] P5 P4 P3 P2 P2' P3'	K_i (pepsin) (nM)[b]	K_i (His-PR) (nM)	K_i (PR) (nM)
CS-I-51 (714)	Iva-Val-Val-Sta-Lys-Iaa	0.72	549 ± 325	466 ± 74
CS-I-22 (611)	Iva-Val-Nle-Sta-Ala-Iaa	0.030	258 ± 24	138 ± 10
CS-I-25 (686)	Iva-Lys-Val-Sta-Ala-Iaa	19.2	10.5 ± 8.3	7.2 ± 0.2
CS-I-27 (794)	Boc-Lys-Val-Val-Sta-Ala-Iaa	0.22	107 ± 6	142 ± 6
CS-I-52 (730)	Iva-Val-Val-Sta-Ala-Lys-OMe	0.10	10,000 ± 5,600	7,200 ± 1,500

[a] Iva, isovaleryl $(CH_3)_2CHCH_2CO$; Nle, norleucine $CH_3(CH_2)_3CH(NH_2)COOH$; Sta, (3*S*,4*S*)-statine $(CH_3)_2CHCH_2CH(NH_2)CH(OH)CH_2COOH$; Iaa, isoamylamine $H_2NCH_2CH_2CH(CH_3)_2$; Boc, isobutyl oxycarboxyl $(CH_3)_3COCO$. [b] Kuzmic et al. (*24*).

Table 3: Inhibition of HTLV-I Protease by HIV-1 Protease Inhibitors

inhibitor (molecular mass)	structure	K_i (HIV-1 PR) (nM)	K_i (PR) (nM)
MES13-099 (407)	nonnitrogen heterocyclic cpd	7[a]	243 ± 88
JG-365 (860)	Ac−Ser-Leu-Asn-Phe[HEA[b]]-Pro-Ile-Val-OMe	0.66 (R/S)[c,d]	6.0 ± 0.6
N-1270 (844)	Arg-Val-Leu** Phe-Glu-Ala-Nle-NH_2[e]	50[f]	127 000 ± 44 000
N-1395 (404)	Ac-Leu-Val-Phe-H	IC_{50} = 900[g]	93 000 ± 29 000
N-1460 (920)	Ser-Gln-Asn-Phe**Pro-Ile-Val-Gln-OH[e]	1000[h]	7300 ± 800
N-1465 (770)	Ac−Thr-Ile-Nle**Nle-Gln-Arg[e]	780[i]	12 700 ± 2 500

[a] Solomon (*22*). [b] HEA, Ψ[CH(OH)CH_2N] hydroxyethylamine. [c] Swain et al. (*25*) and Rich et al. (*26*). [d] R/S, stereoisomer. [e] **, reduced peptide bond Ψ(CH_2NH). [f] Schneider et al. (*31*). [g] Sarubbi et al. (*32*). [h] Heimbach et al. (*33*). [i] Miller et al. (*34*).

measured to determine the relationship of HTLV-I protease to HIV-1 protease. The K_i values that were obtained ranged from 6 nM to 127 μM, but in all cases the K_i values for the inhibition of HTLV-I protease were larger than the K_i values reported for HIV-1 protease (Table 3).

DISCUSSION

HTLV-I, like HIV-1, encodes a protease that is responsible for processing the retroviral precursor proteins. HTLV-I protease cleavage of peptides representing the HTLV-I retroviral processing sites P19/24 and P24/15 yields K_m values of 470 and 310 μM and k_{cat} values of 0.0060 and 0.184 s^{-1}. The P19/24 data was similar to the data obtained by Kobayashi et al. (*15*), but no kinetic data has been previously reported for P24/15. When the P1′ proline of P19/24 (APQVLPVMHPHG) was replaced with Nph, the ability of HTLV-I protease to cleave the modified substrate (APQVLNphVMHPL) was greatly improved. The K_m was reduced, and the k_{cat} increased. The reduction in K_m suggested improved substrate binding which may result from the interaction of the electron-deficient NO_2 group of Nph with electron-rich side chains such as Phe, Tyr, Trp, or His, hydrogen bonding of the NO_2 group to a hydrogen bond donor, or replacement of the cleavage site tertiary amide bond with a secondary amide bond. The increase in k_{cat} suggested a stabilization of the hydrolysis transition state, but the reasons for this effect are not easily explained in the absence of a crystal structure of HTLV-I protease.

Inhibition of HTLV-I protease and His-protease by a series of peptidomimetic inhibitors of pepsin was also tested. It was found that the K_i values for inhibition of HTLV-I protease and His-protease by the CS-I-# series of pepsin inhibitors ranged from 7 nM to 10 μM and that the mature HTLV-I protease K_i values were nearly identical to the His-protease K_i values. In comparison, the K_i values for inhibition of pepsin by the pepsin inhibitors ranged from 0.72 to 19.2 nM with the best pepsin inhibitor being the worst HTLV-I protease inhibitor. The data not only reinforced the observation that mature HTLV-I protease and His-protease are enzymatically similar but also suggested that the peptide binding sites of HTLV-I protease and pepsin are different.

The CS-I-# compounds were designed and synthesized to characterize the effect of charged amino acids on the binding to the mammalian aspartic protease pepsin (*24*). Lysine side chains generally weaken the binding between pepsin and inhibitor when the lysine residue is in the P1 and P3 positions but have little or no effect on binding when placed in other positions. Several of the CS-I-# inhibitors were tested against HTLV-I protease to begin mapping the binding interactions in the enzyme active site to see if significant differences exist between these aspartic proteases. The data in Table 2 show that lysine in P3 produces the best inhibitor of HTLV-I protease and the worst inhibitor of pepsin. Changes in substituents in other positions of the inhibitors also affect the two enzymes differently. Lysine in either P2′ or P3′ has little effect on inhibition of pepsin but clearly weakens the interactions with HTLV-I protease. These trends in the K_i data for HTLV-I protease suggest that a P3 Lys improves inhibitor binding while a P3′ Lys interferes with inhibitor binding.

The positive effect of a P3 Lys on enzyme−ligand interactions was further substantiated by the K_m data for cleavage of the P19/24 and P24/15 substrates. The K_m of P24/15 (KTKVLVVQPK) which contains a P3 Lys was 2-fold lower than the K_m of P19/24 (APQVLPVMHPHG). All of this suggested that there are stabilizing interactions between HTLV-I protease and a P3 Lys and destabilizing interactions between HTLV-I protease and a P3′ Lys. However, if HTLV-I protease is a symmetric dimer like HIV-1 protease, it is surprising that lysines at the symmetrically related P3 and P3′ positions interact with HTLV-I protease so differently. At present, an explanation of this P3/P3′ selectivity will require additional experimental work.

HTLV-I protease is an aspartic acid protease like HIV protease, but protease inhibitors for the treatment of HTLV-I infections and the prevention of HTLV-I-induced leukemia

are unknown. To determine whether HTLV-I protease can be inhibited by HIV-1 protease inhibitors, inhibition of HTLV-I protease by the HIV-1 inhibitors MES13−099 (*22*, *23*), JG-365 (*25*, *26*), N-1270 (*31*), N-1395 (*32*), N-1460 (*33*), and N1465 (*34*) was measured. In all cases the K_i values for inhibition of HTLV-I protease were larger (7−2000 times larger) than the K_i values reported for HIV-1 protease. This suggested that the substrate binding sites of HTLV-I protease and HIV-1 protease are different, but may have some similarities since JG-365 was an inhibitor of both HTLV-I protease and HIV-1 protease. The data also suggested that currently employed HIV protease inhibitors could not be used to treat HTLV-I infections.

With determination of the specificity of HTLV-I protease and investigation of the inhibition of HTLV-I protease by various inhibitors, an understanding of substrate recognition by HTLV-I protease will be obtained. In the future, we hope to identify potent inhibitors of HTLV-I protease for possible treatment of HTLV-I infections.

NOTE ADDED IN PROOF

Pettit et al. (*35*) recently reported that Gag protein processing in Rhesus monkey cells infected with HTLV-I was not inhibited by the HIV protease inhibitors, indinavir, saquinavir, ritonavir, and nelfinavir.

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