SCHEME 2 Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii), Fmoc–AA–OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iii), Boc–Ser–OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iv), Fmoc–Gly–OH (3.0 eq), DIPCDI (3.0 eq), DMAP (0.2 eq), CH_2Cl_2, 16 h × 2; (v), TFA–*m*-cresol–thioanisole–H_2O (92.5:2.5:2.5:2.5), 90 min; (vi), NH_3I (20 eq), dimethylsulfide (20 eq), TFA:H_2O (2:1), 60 min, 0°C; (vii), preparative HPLC (a linear gradient of CH_3CN in 0.1% aqueous TFA).

O-acylation, since the adjacent Gly[25] does not epimerize during ester bond formation (Figure 10).

As depicted in Scheme 2, Fmoc–Ala–*O*–chlorotrityl resin (0.465 mmol/g, **14**) was employed and Fmoc-

protected amino acids were sequentially coupled using the DIPCDI–HOBt method (2 h) after the removal of each Fmoc group with 20% piperidine–DMF (20 min) to give peptide resin **15**. After Boc–Ser–OH
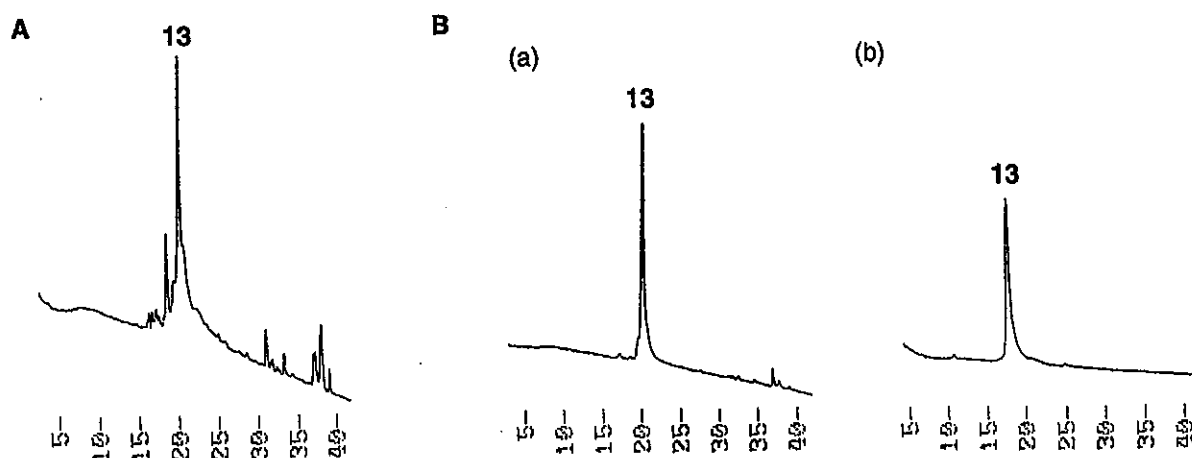

FIGURE 11 HPLC profiles of (A) crude and (B) purified 26-AIAβ1–42 (**13**). Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN, 40 min for A and B-a, 25–55% CH_3CN, 60 min for B-b) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40°C), detected at 230 nm.

was introduced to 15, the obtained 16 was coupled with Fmoc–Gly–OH at the β-hydroxy group of Ser using the DIPCDI–DMAP method in CH_2Cl_2 to obtain ester 17. The compound 26-AIAβ1–42–resin (18) was obtained through the coupling of additional amino acid residues in the conventional manner. Finally, 26-AIAβ1–42 (13) was obtained as a major product by treatment with TFA–*m*-cresol–thioanisole–H_2O (92.5:2.5:2.5:2.5) for 90 min followed by NH_3I–dimethylsulfide for 60 min in TFA:H_2O (2:1).

In HPLC analysis of crude products (Figure 11A), Aβ1–25 (DAEFRHDSGYEVHHQKLVFFAEDVG) was not observed as a by-product, although a very low rate (1.6%, HPLC yield) of Aβ26-42 (SNKGAI-IGLMVGGVVIA) was detected. This indicates that (1) the esterification of the β-hydroxy group of Ser was successfully completed on the solid support and (2) the formed ester bond between Gly and Ser was stable in both piperidine and TFA. The crude O-acyl isopeptide 13 was dissolved in hexafluoroisopropanol, applied to preparative HPLC, and eluted using 0.1%

aqueous TFA–CH_3CN. Since 13 was eluted as a narrow single peak, it could easily be purified using preparative scale HPLC to give pure 13 (Figure 11B) as TFA salt with a total isolated yield of 33.6%, calculated from the original loading of chlorotrityl resin. This yield was higher than that obtained in the synthesis of 12 by standard Fmoc-based SPPS (7.2%). Since 12 was eluted as a broad peak in preparative scale HPLC purification, it was laborious to isolate 12 from impurities as reported.[56-59] In addition, in the synthesis of 13, no conversion to 12 was observed.

The water solubility of 13 (TFA salt) was 15 mg mL^{-1}, 100-fold higher than that of Aβ1–42 (12, 0.14 mg mL^{-1}). Interestingly, as a slight modification of the peptide chain by inserting one ester bond could drastically increase the solubility of the insoluble original peptide with 42 residues, this suggests that the O-acyl isopeptide totally breaks the secondary structures responsible for the insolubility of the original peptide. As we demonstrated that the O-acyl isopeptide could suppress the unfavorable nature of
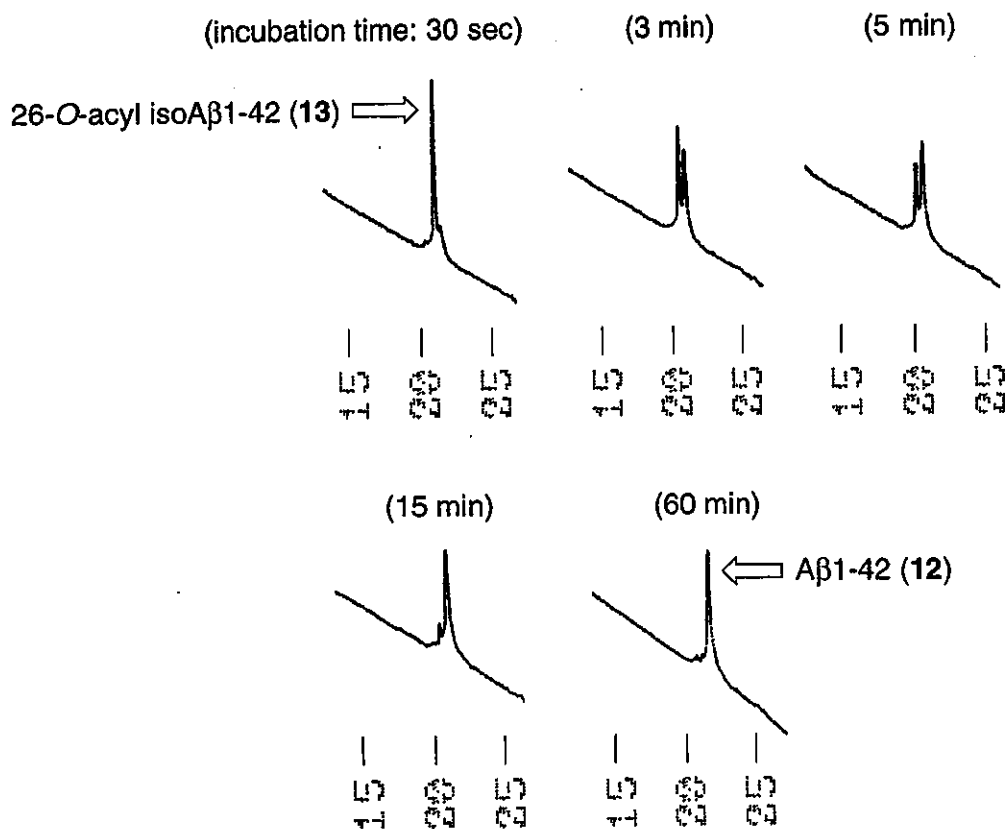

FIGURE 12   HPLC profiles of the conversion of 26-AIAβ1–42 (13) to Aβ1–42 (12) via O–N intramolecular acyl migration in PBS (pH 7.4, 25°C). Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40°C), detected at 230 nm.

difficult sequence-containing pentapeptides in the previous study,[40] this result in the synthesis of **13** indicates that this method is a powerful strategy for increasing the solubility of even large peptides.

As shown in Figure 12, purified **13** was completely converted to Aβ1–42 (**12**) at room temperature in PBS (pH 7.4) with no side reaction. This migration was rapid with a half-life of 2.6 min, while the TFA salt of **13** was stable at 4°C for at least 30 days in either a solid state or a DMSO solution. Moreover, slower migration was observed at pH 4.9 (PBS) with a half-life of 3 h and no migration at pH 3.5 (acetate buffer) after incubation for 3 h. This rapid migration under physiological conditions enables the production of an intact monomer, Aβ1–42, in situ to investigate the inherent biological function of Aβ1–42 in AD. The conversion of **13** (TFA salt) in water for 48 h at room temperature followed by lyophilization yielded Aβ1–42 (**12**) quantitatively as TFA salt with >95% purity.

This result demonstrates that this O–N acyl migration strategy via O-acyl isopeptides is applicable for the synthesis of large peptides. In particular, it is noteworthy that only one insertion of the isopeptide structure into the sequence of 42-residue peptide can suppress the unfavorable nature of its difficult sequence. Therefore, the O–N acyl migration strategy can be applied to larger difficult sequence-containing peptides than Aβ1–42 as a general method. In addition, rapid migration of O-acyl isopeptides to intact Aβ1–42 under physiological conditions (pH 7.4) was observed while it was stable under storage conditions. Hence, our strategy not only overcomes the solubility problem in the synthesis of Aβ1–42, but also provides a novel tool for the biological evaluation system in AD research, in which 26-O-acyl isoAβ1–42 can be stored in a solubilized form before use and rapidly produces intact Aβ1–42 in situ during biological experiments.

PERSPECTIVE FOR THE FUTURE

Half a century has passed since the N–O acyl migration of Ser/Thr-containing peptides in strong acids treatment was reported.[1-3] This classical side reaction has recently been revived as a powerful key reaction in the field of modern medicinal chemistry in the development of sophisticated prodrugs. In addition, this reaction was introduced again in peptide chemistry for the synthesis of difficult sequence-containing peptides, which are a major drawback in automated solid-phase peptide synthesis. These applications contribute to medical science to understand the function

and mechanism of bioactive peptides and provide new prodrug candidates. In addition, this strategy, based on the economical reaction in aqueous media, will develop further in 21st century science such as organic chemistry by providing new water-soluble substrates required in green chemical reactions.

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ELSEVIER

Identification of peptidomimetic HTLV-I protease inhibitors containing hydroxymethylcarbonyl (HMC) isostere as the transition-state mimic

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Abstract—Towards the development of chemotherapy for the infection by human T-cell leukemia virus type I (HTLV-I), we have established evaluation systems for HTLV-I protease (PR) inhibitors using both recombinant and chemically synthesized HTLV-I PRs. Newly synthesized substrate-based inhibitors containing hydroxymethylcarbonyl (HMC) isostere showed potent anti-HTLV-I PR activity.
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1. Introduction

The human T-cell leukemia virus type I (HTLV-I) is a retrovirus that has been clinically associated with adult T-cell leukemia (ATL)[1] and HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP).[2,3] Estimations in 1997 revealed that between one and two million people were infected with HTLV-I in Japan where the virus is most prevalent in the world.[4] Since the most recent study reported that increased HTLV-I proviral load in central nervous system is a strong predictor for the development of HAM/TSP,[5] effective anti-HTLV-I agents might contribute to suppress the progression of HAM/TSP. However, such chemotherapeutic agents based on the specific anti-HTLV-I activity have not been developed.

HTLV-I encodes a virus-specific aspartic protease (PR) responsible for processing the *gag* and *gag-pro-pol* polyproteins leading to the proliferation of the retrovirus.[6] Since this process is essential for the retroviral replication, it is suggested that HTLV-I PR is one of the major targets to develop the specific anti-HTLV-I agents. The inhibitors containing statine or hydroxyethylamine isostere were already reported.[7] This idea is also supported by the findings that the inhibitors of HIV PR, which is a similar aspartic protease as HTLV-I PR, made a significant contribution to the successful treatments of AIDS.[8] For the last decade, we have developed HIV PR inhibitors, named 'KNI compounds', based on the concept of 'substrate transition-state mimic',[9] and found that an α-hydroxy-β-amino acid derivative, allophenylnorstatine (Apns), which has a hydroxymethylcarbonyl (HMC) isostere (Fig. 1), provided a unique interaction with the active site of HIV PR essentially similar to that of the substrates,[9] and the KNI compounds having Apns exhibited highly potent inhibition of HIV-1 replication. Furthermore, our recent studies using HMC compounds demonstrated effective inhibition of the malaria parasite aspartic protease, plasmepsin II,
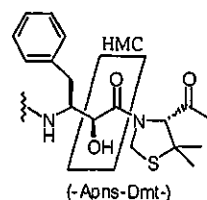
Figure 1. Structure of HMC isostere.

A

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        10         20         30         40
MGHHHHHHHH HHSSGHIDDD DKHMPVIPLD PARRPVIKAQ
        50         60         70         80
VDTQTSHPKT IEALLDTGAD MTVLPIALFS SNTPLKNTSV
        90        100        110        120
LGAGGQTQDH FKLTSLPVLI RLPFRTTPIV LTSCLVDTKN
       130        140
NWAIIGRDAL QQCQGVLYLP EAKGPPVIL
```

B

```
        10         20         30         40
PVIPLDPARR PVIKAQVDTQ TSHPKTIEAL LDTGADMTVL
        50         60         70         80
PIALFSSNTP LKNTSVLGAX XQTQDHFKLT SLPVLIRLPF
        90        100        110        120
RTTPIVLTSA LVDTKNNWAI IGRDALQQAQ GVLYLPEAKG
PPVIL
```

Figure 2. The amino acid sequences of prepared HTLV-I PRs. (A) recombinant HTLV-I PR. Underline: His-tag sequence, boldface: mature HTLV-I PR sequence. (B) synthetic [(NH$_2$CH$_2$CH$_2$-SCH$_2$CO)$^{60-61}$, Ala^{90,109}]-HTLV-I PR. Underline: substitution sites from Cys to Ala. XX: thioether linkage (-NHCH$_2$CH$_2$SCH$_2$CO-).

suggesting that this motif can be applicable to other aspartic proteases as a universal inhibitory machinery.[10] Based on these backgrounds, to obtain potent HTLV-I PR inhibitors, it would be significant to know whether the existing HIV PR inhibitors with Apns are effective on HTLV-I PR inhibition. Hence, in the present study, we established a screening system for HTLV-I PR inhibitory activity using both recombinant and chemically synthesized HTLV-I PRs and evaluated the existing and newly synthesized compounds having the HMC motif.

2. Expression and synthesis of HTLV-I PRs

Although several studies for the expression of HTLV-I PR in *E. coli*[11] and its chemical synthesis,[12] which are useful for screening HTLV-I inhibitors, were reported, they seem practically difficult in our laboratory. We independently prepared both recombinant (rec) and chemically synthesized (syn) HTLV-I PRs (Fig. 2) by easy ways to establish a screening system. In the preparation of recombinant PR in *E. coli*, the HTLV-I PR gene[13] was inserted down the stream of a decahistidine-containing leader sequence under the control of T7 promoter of pET19b (Novagen). Then, the constructed plasmid was introduced into *E. coli* BL21(DE3)-pLys S cells and the gene expression was induced by the addition of IPTG.[14] The cultured cells were collected by centrifugation and resuspended in lysis buffer A (10 mM Tris containing 100 mM NaH$_2$PO$_4$, 1 mM EDTA, 10 mM 2-mercaptoethanol and 8 M urea; pH 8.0). After the bacterial cell lysate was centrifuged, the obtained supernatant was applied to a His-Bind affinity column[15] to give the pure PR. From the SDS-PAGE analysis,[16] the rec-PR showed a single band with a molecular size of approximately 16 kDa.

[NHCH$_2$CH$_2$SCH$_2$CO$^{60-61}$, Ala^{90,109}]-HTLV-I PR was synthesized using stepwise Fmoc-based solid phase peptide synthesis (SPPS) followed by thioether forming ligation (Fig. 2, Scheme 1).[17] Briefly, a mercaptoethylamide peptide segment, HTLV-I PR (1-59)-NHCH$_2$CH$_2$SH 1, was prepared starting from an Fmoc-AEDI-O-Clt-resin.[18,19] The SPPS was achieved by an ABI 431A synthesizer with a standard DCC-HOBt protocol, and then the
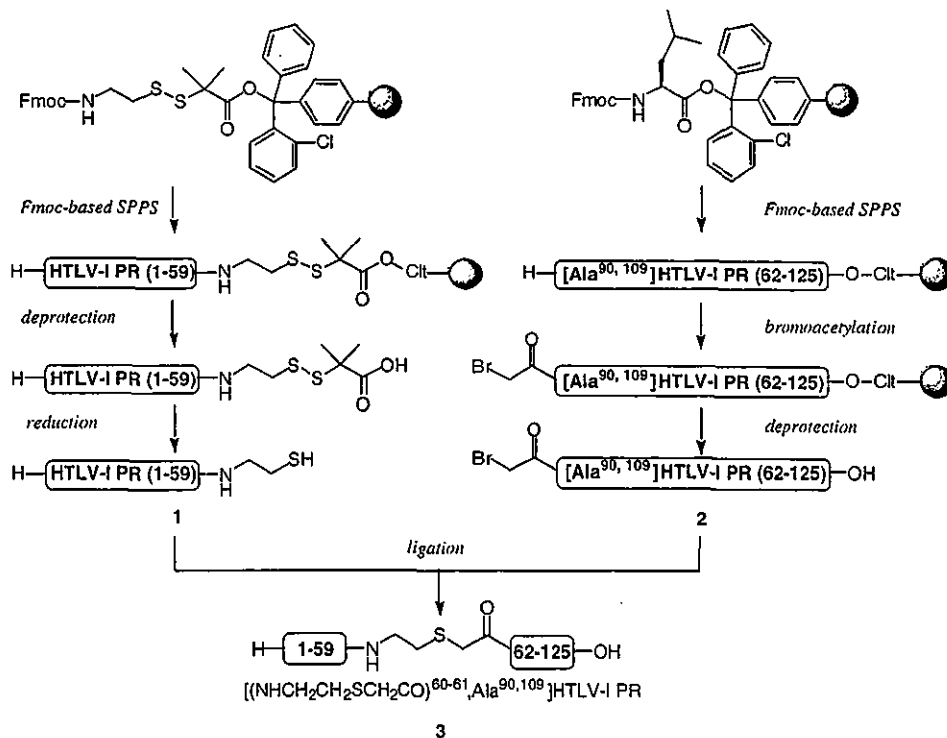
Scheme 1. Synthetic scheme for an HTLV-I PR analog 3 using thioether-forming ligation.

peptide segment containing a linker moiety was cleaved by HF–dimethyl sulfide–*m*-cresol (3:6:1) (0 °C, 1 h). The treatment of the resultant with dithiothreitol (DTT) in 6 M guanidine·HCl containing 200 mM Tris (pH 8.5) gave the desired peptide as the main product. The crude peptide 1 was purified by RP-HPLC and characterized by amino acid analysis of its hydrolysate and MALDI-TOF MS.[20] Another segment, BrCH$_2$CO-[Ala^{90,109}]-HTLV-I PR (62–125) 2 was also prepared by the conventional SPPS, and bromoacetylation of the N-terminal amino group was carried out using bromoacetic acid and 1,3-diisopropylcarbodiimide.

Obtained protected peptide resin was deprotected and cleaved from resin with HF–*m*-cresol (0 °C, 1 h), followed by the purification using RP-HPLC, and characterization by amino acid analysis and MALDI-TOF MS.[21]

The ligation of the two segments 1, 2 was carried out in 6 M guanidine·HBr-containing 200 mM Tris (pH 8.5) at

room temperature under an Ar atmosphere for 3 h with vigorous stirring (Fig. 3). Then, the treatment with DTT reduced the undesirable disulfide dimer of peptide segment 1, which was removed by the gel filtration with Hiload 16/60 superdex 75 prep grade in 8 M urea-containing 0.5 M KH$_2$PO$_4$. The purified protein was characterized as [NHCH$_2$CH$_2$SCH$_2$CO$^{60-61}$, Ala^{90,109}]-HTLV-I PR 3 by amino acid analysis and MALDI-TOF MS[22] and showed a single peak on RP-HPLC analysis.

Both protein solutions were concentrated using a centrifugal filter (Centricon Plus-20, Millipore) followed by the replacement with 7.5 M guanidine·HCl and dialysis with a large excess amount of 20 mM PIPES, pH 7.0, containing 2 mM DTT, 1 mM EDTA, 150 mM NaCl and 10% glycerol at 25 °C for 16 h to fold into the matured protein structures with the PR activity.[11c] Then, the solution was concentrated again using the same centrifugal filter to a protein concentration greater than 5 μM, and this enzyme solution was used for the HTLV-I PR inhibition assay.
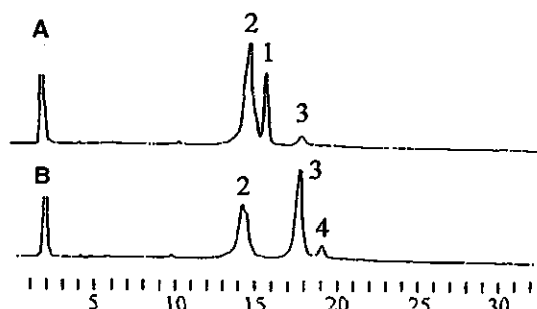

Figure 3. HPLC profiles of ligation reaction of peptide 1 (1.0 equiv) and 2 (1.5 equiv) at 0 h (A) and 3 h (B). The peaks 3 and 4 show [NHCH$_2$CH$_2$SCH$_2$CO$^{60-61}$, Ala^{90,109}]-HTLV-I PR and disulfide dimer of peptide 1, respectively. HPLC was performed using a C18 reverse phase column with a liner gradient of 35–55% CH$_3$CN in 0.1% aqueous TFA over 30 min at a flow rate of 0.9 mL/min with a detection at UV 230 nm.

3. Synthesis of HTLV-I PR inhibitors

Based on an HTLV-I cleavage site, octapeptides 10–13 with the HMC motif were newly designed and synthesized manually using a conventional Fmoc-based SPPS. The peptide resins were cleaved by TFA–*m*-cresol–thioanisole–H$_2$O (85:5:5:5), and the octapeptides were purified by RP-HPLC and characterized by MALDI-TOF MS before biological evaluation. Synthesis of 5–9 was previously reported.[23–25]

4. Results and discussion

Both the recombinant and synthetic HTLV-I PR analogs specifically hydrolyzed synthetic peptide substrates, APQVL*PVMHP (p19/24), KTKVL*VVQPK (p24/15)

Table 1. Inhibition of HTLV-I PRs and HIV-1 PR by HMC compounds

Compound	Structure								Inhibition (%)				
	P4	P3	P2	P1	P1′	P2′	P3′	P4′	rec-HTLV-I PR		syn-HTLV-I PR		HIV-1 PR[b]
									At 100 μM	At 5 μM	At 100 μM	At 5 μM	At 50 nM
5 (KNI-272)	iQoa-Mta-Apns-Thz -NHBu′								10	—	39	—	97
6 (KNI-577)	Bz(3-OH, 2-Me)-Apns-Dmt-NHBu′								38	—	57	—	88
7 (KNI-727)	Pac(diMe)-Apns-Dmt-NHBu′								43	—	32	—	96
8 (KNI-764)	Bz(3-OH, 2-Me)-Apns-Dmt-NHBzl(Me)								31	—	79	—	96
9 (KNI-840)	Pac(diMe)-Apns-Dmt-NHBzl(Me)								19	—	11	—	98
10 (KNI-10159)	H-Pro-Gln-Val-Anst -Pro- Val-Met-His-OH								93	43	—	58	3
11 (KNI-10160)	H-Pro-Gln-Val-Anst -Dmt-Val-Met-His-OH								100	63	—	76	37
12 (KNI-10161)	H-Pro-Gln-Val-Apns-Pro- Val-Met-His-OH								94	54	—	59	6
13 (KNI-10162)	H-Pro-Gln-Val-Apns-Dmt-Val-Met-His-OH								100	66	—	80	58
Ritonavir	—[a]								20	—	20	—	100
Pepstatin A	Iva-Val-Val- Sta -Ala- Sta -OH								—	17	—	15	23

[a]See Ref. 8. iQoa, isoquinolyloxyacetyl; Mta, methylthioalanine; Thz, 1,3-thiazolidine-4-carboxylic acid; NHBu′, *tert*-butylamide; Bz(3-OH, 2-Me), 3-hydroxy-2-methylbenzoyl; Pac(diMe), 2,6-dimethylphenoxyacetyl; NHBzl(Me), 2-methylbenzylamide; Iva, isovaleryl; Sta, (3*S*,4*S*)-statine; Anst, allonorstatine, [(2*S*,3*S*)-3-amino-2-hydroxy-5-methylhexanoic acid]; Apns, allophenylnorstatine, [(2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid]; Dmt, (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid. The modified p19/24, APQVL*NphVMHPL, was used as a substrate.
[b]HIV-1 PR inhibition was determined by monitoring the fluorescence change (305 nm, λ_{ex} = 275 nm) associated with the cleavage of the fluorogenic substrate, H-Lys-Ala-Arg-Val-Tyr*Nph-Glu-Ala-Nle-NH$_2$. Nph, *p*-nitrophenylalanine.

Table 2. K_i values for pepstatin A and 13

Inhibitor	K_i (rec PR)[a] (μM)	K_i (syn PR)[a] (μM)
Pepstatin A	14.6 ± 3.8	36.5 ± 5.0
13 (KNI-10162)	3.9 ± 0.7	2.0 ± 0.5

[a] Values represent the mean of three experiments ± SEM.

and APQVL*NphVMHPL (the modified p19/24) (* indicate scissile bond),[26,27] and pepstatin A, which is known as a typical aspartyl PR inhibitor, showed a moderate inhibitory activity with K_i values of 14.6 and 36.5 μM against rec- and syn-HTLV-I PRs, respectively (Tables 1 and 2).[28,29] These K_i values were almost equal to that of previously reported recombinant HTLV-I PR ($K_i = 17$ μM).[11a] These results suggested that the both rec- and syn-HTLV-I PRs are enzymatically similar to the native HTLV-I PR even they have modifications in their structures and the screening system using these PRs is effective to evaluate HTLV-I PR inhibitors.

The inhibitory activities of the HMC compounds are summarized in Tables 1 and 2.[28,29] Compounds 5–9, which were developed as potent HIV PR inhibitors, showed weak inhibitory activity against the HTLV-I PRs. Ritonavir, which is a clinically used HIV PR inhibitor, also showed weak activity. These results indicated that HIV PR inhibitors were not effective for the inhibition of HTLV-I PR, suggesting that the recognition of inhibitors is different between the two viral proteases despite the relatively similar natural substrate sequences. However, this result is compatible with the findings that the most synthetic HIV-1 PR substrates were not cleaved by HTLV-I PR, except for IRKIL*FLDG from the RT/IN site.[11c] Hence, to obtain effective HMC compounds with a potent anti-HTLV-I PR activity, we designed and synthesized new inhibitors 10–13 consisting of the P4–P4′ sites of the HTLV-I PR cleavage sequence at the p19/24 (MA/CA) site in the gag region. These octapeptides showed a potent inhibitory activity with 50% to 70% inhibition at 5 μM and a relatively decreased anti-HIV PR activity. The K_i values of 13 were 3.9 and 2.0 μM against rec- and syn-HTLV-I PRs, respectively (Table 2), which were at least 3-fold more potent than pepstatin A. The similar anti-HTLV-I PR activity shown among these peptides was probably due to the conservative modification only at the P1 and P1′ sites. These results suggest that the HMC motif functions as the inhibitory machinery of HTLV-I PR, but the recognition of the other P and P′ sites are also important for the effective interaction leading to the potent HTLV-I PR inhibition, since weak inhibitors 6–9 also possess the same Apns-Dmt core structure as potent compound 13. Extensive modifications focusing on the size of the molecule and each side chain structure based on the present octapeptides are underway.

In conclusion, we have established an evaluation system for HTLV-I PR inhibitors using both recombinant and chemically synthesized HTLV-I PRs and found that newly synthesized substrate-based inhibitors with the HMC motif showed potent anti-HTLV-I PR activity.

Acknowledgements

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15. The supernatant was incubated with 2 mL of Ni-NTA agarose (Qiagen) for 1 h at 25 °C with shaking. The Ni-NTA agarose was packed into an empty column and washed three times with 5 mL of buffer B (buffer A, pH 6.3). The protein was eluted with buffer C (buffer A, pH 4.5).
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peptide 1 (m/z 6319.89 (M+H$^+$), calcd for C$_{286}$H$_{465}$-N$_{73}$O$_{83}$S$_2$: 6319.41).

21. Standard amino acid analysis of peptide 2: Asx (5.2), Thr (3.9), Ser (1.2), Glx (5.9), Gly (3.4), Ala (5.3), Val (4.7), Ile (4.0), Leu (10.0), Tyr (0.9), Phe (1.4), Lys (3.0), His (0.7), Arg (2.5), Pro (5.9). MALDI-TOF MS of peptide 2 (m/z 7199.02 (M$^+$), calcd for C$_{328}$H$_{531}$BrN$_{86}$O$_{90}$: 7199.16).

22. Standard amino acid analysis of peptide 3: Asx (10.0), Thr (11.1), Ser (5.2), Glx (9.2), Gly (5.3), Ala (11.0), Val (8.5), Met (0.9), Ile (6.6), Leu (17.2), Tyr (1.0), Phe (3.8), Lys (5.9), His (1.8), Trp (0.5), Arg (4.5), Pro (12.5). MALDI-TOF MS of peptide 3 (m/z 13399.9 (M+H$^+$), calcd for C$_{607}$H$_{998}$N$_{161}$O$_{174}$S$_2$: 13399.6).

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27. The activities of rec- and syn-HTLV-I PRs were determined by measuring the rate of hydrolysis of the synthetic substrates, APQVL*PVMHP (p19/24), KTKVL*VVQPK (p24/15) and APQVL*NphVMHPL (the modified p19/24). The enzyme reactions contained 2 μM protease (as a dimer) and 20–200 μM substrates in 200 mM sodium citrate buffer, pH 5.3, containing 1 mM DTT, 1 M NaCl, 5 mM EDTA and 6% glycerol, which were incubated at 37 °C for various periods, and then terminated by the addition of 20% TCA. The produced peptide fragments were measured by HPLC using a C18 column with a linear gradient of 4–28% (for p19/24), 0–18% (for p24/15) and 5–35% (for the modified p19/24) CH$_3$CN containing 0.1% TFA for 12, 14 and 15 min, respectively, monitored at 215 nm. The concentration of each fragment was calculated with a standard curve.

28. All inhibitors were dissolved in DMSO to make a 5 mM stock solution. Final concentrations in the inhibitor assay were 2.0 μM HTLV-I PR, 200 μM substrate (the modified p19/24), 200 mM sodium citrate buffer, pH 5.3, containing 1 mM DTT, 1 M NaCl, 5 mM EDTA, 6% glycerol and 2% DMSO containing 5 or 100 μM inhibitor at 37 °C for 6 h. The residual protease activity was analyzed by the same way.

29. Inhibition constants (K_i) were determined by the method of Dixon.[30] The reactions were incubated at 37 °C for 30 min. The modified p19/24, APQVL*NphVMHPL, was used as a substrate.

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Search for Substrate-Based Inhibitors Fitting the S₂' Space of Malarial Aspartic Protease Plasmepsin II

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Abstract: Plasmepsin (Plm) has been identified as an important target for the development of new antimalarial drugs, since its inhibition leads to the starvation of Plasmodium falciparum. A series of substrate-based dipeptide-type Plm II inhibitors containing the hydroxymethylcarbonyl isostere as a transition-state mimic were synthesized. The general design principle was provision of a conformationally restrained hydroxyl group (corresponding to the set residue at the P₂' position in native substrates) and a bulky unit to fit the S₂' pocket. Copyright © 2004 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimalarial drug; aspartic protease inhibitor; plasmepsin; transition-state mimic;
allophenylnorstatine

INTRODUCTION

Malaria parasites use hemoglobin as a source of nutrients during their growth and maturation in erythrocytes. The prevention of this process is thought to be a key target for the development of a new drug against malaria. Recently, it became clear that malaria parasites encode several proteases that are essential components of their hemoglobin-degradation pathway [1]. For example, in the food vacuole of Plasmodium falciparum, four aspartic proteases, plasmepsin (Plm) I, II, and IV and

histo-aspartic protease (HAP), whose amino acid sequences are highly conserved, are involved in the pathway [2,3]. Since it is reported that the inhibition of Plm I and II which initiate the hemoglobin-degradation leads to starvation of the parasites [4], these enzymes are the targets for the development of new antimalarial drugs and several inhibitors, including our compounds [5,6], have already been reported [7–12].

Our previous study on the development of new antimalarial drugs focused on Plm II as a target enzyme for structure-based drug design, since Plm II can be efficiently expressed in E. coli, and its high-resolution structure has been determined by x-ray crystallography [8]. Our HIV protease (PR) inhibitor study developed a series of substrate-based peptidomimetic inhibitors containing an allophenylnorstatine [Apns; (2S,3S)-3-amino-2-hydroxy-4-phenylbutyric acid] with a hydroxymethylcarbonyl (HMC) isostere as an ideal

Table 1 Substrate Cleavage Sites (*) of Plasmepsin II and HIV-1 Protease

Enzyme	Cleavage site	- P4	P3	P2	P1*P1'	P2'	P3'	P4' -
Plasmepsin II	α33/34	- Glu	Arg	Met	Phe*Leu	Ser	Phe	Pro -
	α108/109	- Leu	Leu	Val	Thr*Leu	Ala	Ala	His -
	α136/137	- Ser	Thr	Val	Leu*Thr	Ser	Lys	Tyr -
	β32/33	- Gly	Arg	Leu	Leu*Val	Val	Tyr	Pro -
HIV-1 protease	p17/p24	- Gln	Arg	Gly	Tyr*Pro	Ile	Val	Gln -
	p24/p1	- Ala	Arg	Val	Leu*Ala	Glu	Ala	Met -
	p1/p9	- Ala	Thr	Ile	Met*Met	Gln	Arg	Gly -
	p9/p6	- Pro	Gly	Asn	Phe*Leu	Gln	Ser	Arg
	TF/PR	- Ser	Phe	Asn	Phe*Pro	Gln	Ile	Thr
	PR/RT	- Thr	Leu	Asn	Phe*Pro	Ile	Ser	Pro
	RT/RN	- Ala	Glu	Thr	Phe*Tyr	Val	Asp	Gly
	RN/IN	- Arg	Lys	Ile	Leu*Phe	Leu	Asp	Gly

TF, transframe protein; PR, protease; RT, reverse transcriptase; RN, ribonuclease H; IN, integrase.

KNI-727
K_i 0.07 μM (Plm II)
K_i 1.3 μM (Cat D)
Selectivity 22.4
EC_{50} 10 μM

KNI-764
K_i 0.03 μM (Plm II)
K_i 0.2 μM (Cat D)
Selectivity 6.7
EC_{50} 5.7 μM

KNI-840
K_i 0.02 μM (Plm II)
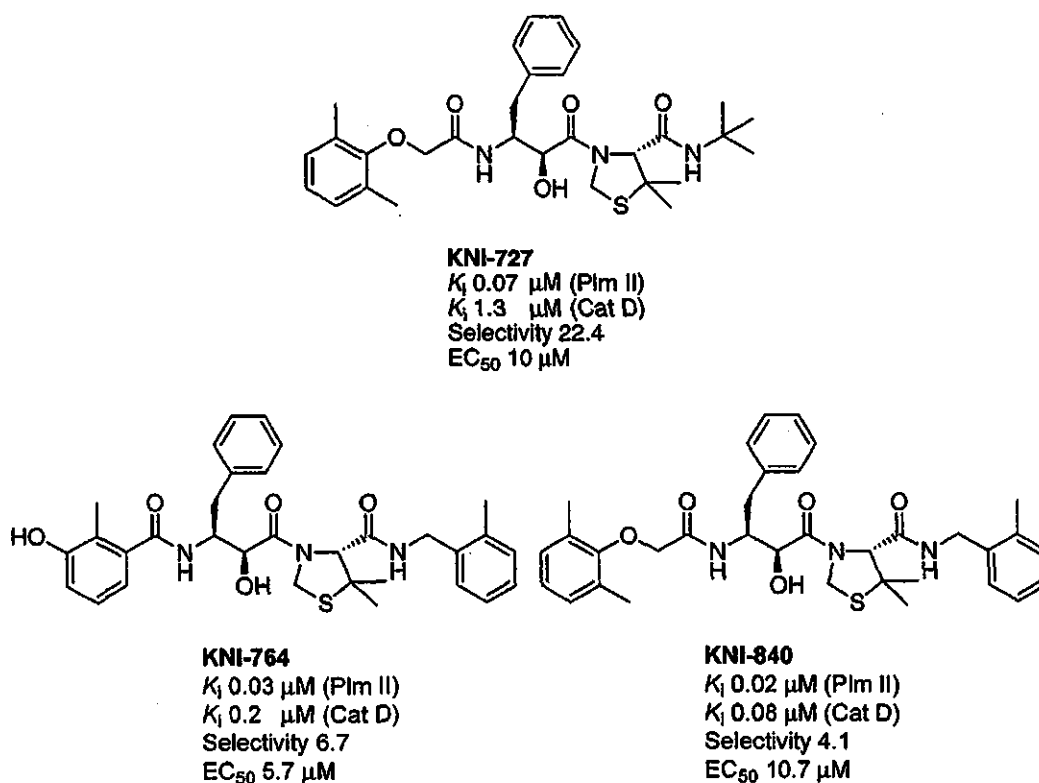K_i 0.08 μM (Cat D)
Selectivity 4.1
EC_{50} 10.7 μM

Figure 1 Structures of KNI compounds.

transition-state mimic [13–18]. Since the substrate recognition profiles of Plm I and II [19] are similar to that of HIV-1 PR (Table 1), and Phe[33]-Leu[34] in the α-chain which is a primary cleavage site in hemoglobin-degradation exhibits a similar

structure to the Apns-containing scaffold, it was hypothesized that HIV-1 PR inhibitors would be effective against Plms, so the inhibitory activity of 12 selected HIV PR inhibitors were evaluated against Plm II [5]. As predicted, these substrate

transition-state mimic compounds are capable of inhibiting Plm II. Among them, KNI-727 [14–16], –764 [17–18] and –840 [16] which contain Apns-Dmt [Dmt = dimethylthioproline; (R)-5,5-dimethyl-1,3-thiazolidinecarboxylic acid] at the P_1-P_1' positions exhibited potent Plm II inhibitory activity with K_i values of 0.07, 0.03 and 0.02 μM, respectively (Figure 1). The Apns-Dmt scaffold is based on the substrate Phe-Leu (P_1-P_1') scissile site transition state; that is, Apns is the Phe-transition state mimic and Dmt is the conformational constrained cyclized Leu-mimic. Interestingly, KNI-727 also exhibited a 23-fold higher potency against Plm II compared with highly homologous human cathepsin D (Cat D). Moreover, these three compounds also exhibited antimalarial activity in cultures of red blood cells infected with P. falciparum with EC_{50} values of 5–10 μM (Figure 1) [5]. This result suggests that Plm II inhibitors based on the HMC-type substrate transition-state mimic are potential antimalarial drugs. In further screening, KNI-10006, which has an (1S,2R)-1-amino-2-indanol at the P2' position, was found to inhibit Plm II with a remarkably high affinity [6–20].

This paper describes the SAR of Plm II inhibitors based on the substrate transition-state mimic. Sixteen compounds (4a–p) were designed and synthesized in which the P_2' position of KNI-727 is modified (Figure 2). Since native substrates contain a Ser residue at the P_2' position (Table 1), a hydroxyl group was introduced with a fixed conformation and a bulky unit to fit the space of the S_2' pocket of Plm II. The enzyme inhibitory activities of these synthetic compounds were evaluated.

MATERIALS AND METHODS

Synthesis

The dipeptidic compounds (4a–p) were prepared by the usual Boc strategy in the liquid phase (Scheme 1). Coupling of Boc-Dmt-OH [17] with the free amino group of the P_2' ligands yielded compounds 2a–p. The Boc group was deprotected with 4N HCl/dioxane, followed by coupling with Boc-Apns-OH [17] using EDC in the presence of HOBt, resulting in protected compounds 3a–p. Finally, deprotection of 3a–p and coupling with 2,6-dimethylphenoxyacetic acid using BOP yielded the desired dipeptide-type analogs, 4a–p. Compounds 4i and 4j were separated by column chromatography from a cis-mixture, but the configurations of each diastereomer have not been established yet.

Enzyme Inhibition Assay

Recombinant HIV-1 PR was purchased from Bachem AG, Bubendorf, Switzerland. HIV PR substrate [H-Lys-Ala-Arg-Val-Tyr-Phe(p-NO_2)-Glu-Ala-Nle-NH_2] was synthesized by a conventional solid-phase method. The determination of HIV-1 PR inhibitory activity of the test compounds at 50 nM was based on the inhibition of cleavage of the HIV PR substrate by using recombinant HIV-1 PR (2 μg/ml) in 50 mM MES-NaOH (pH 5.5) containing 2.5 mM DTT, 1 mM EDTA-2Na, 0.2% Nonidet P-40 and 15% glycerol. After incubation for 15 min at 37 °C, the reaction was terminated by the addition of 1N HCl, and the amount of N-terminal cleavage fragment produced was measured by HPLC.
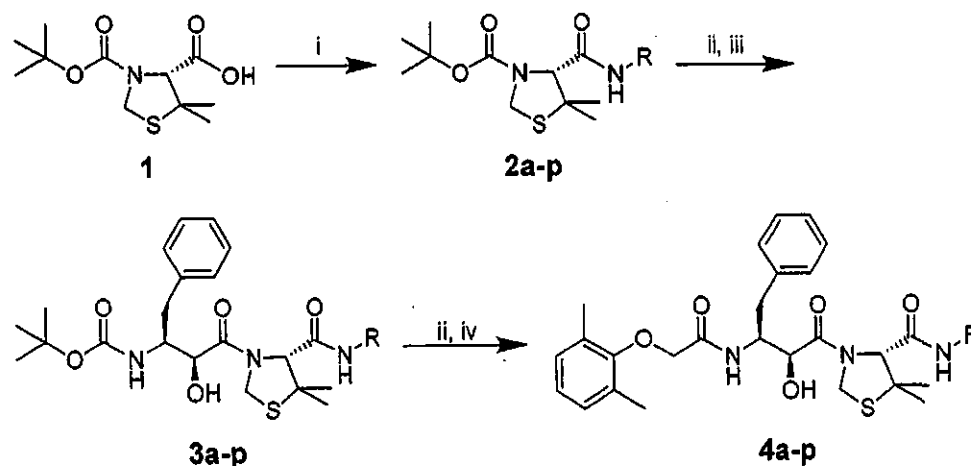
Scheme 1   Reagents and conditions: (i) R-NH_2, TEA, BOP, DMF, rt, 18 h; (ii) anisole, 4N-HCl/dioxane, rt, 1 h; (iii) Boc-Apns-OH, HOBt, EDC, TEA, DMF, rt, 18 h; (iv) TEA, 2,6-dimethylphenoxyacetic acid, BOP, DMF, rt, 18 h.

Figure 2 Structures of synthetic KNI-727 analogs.
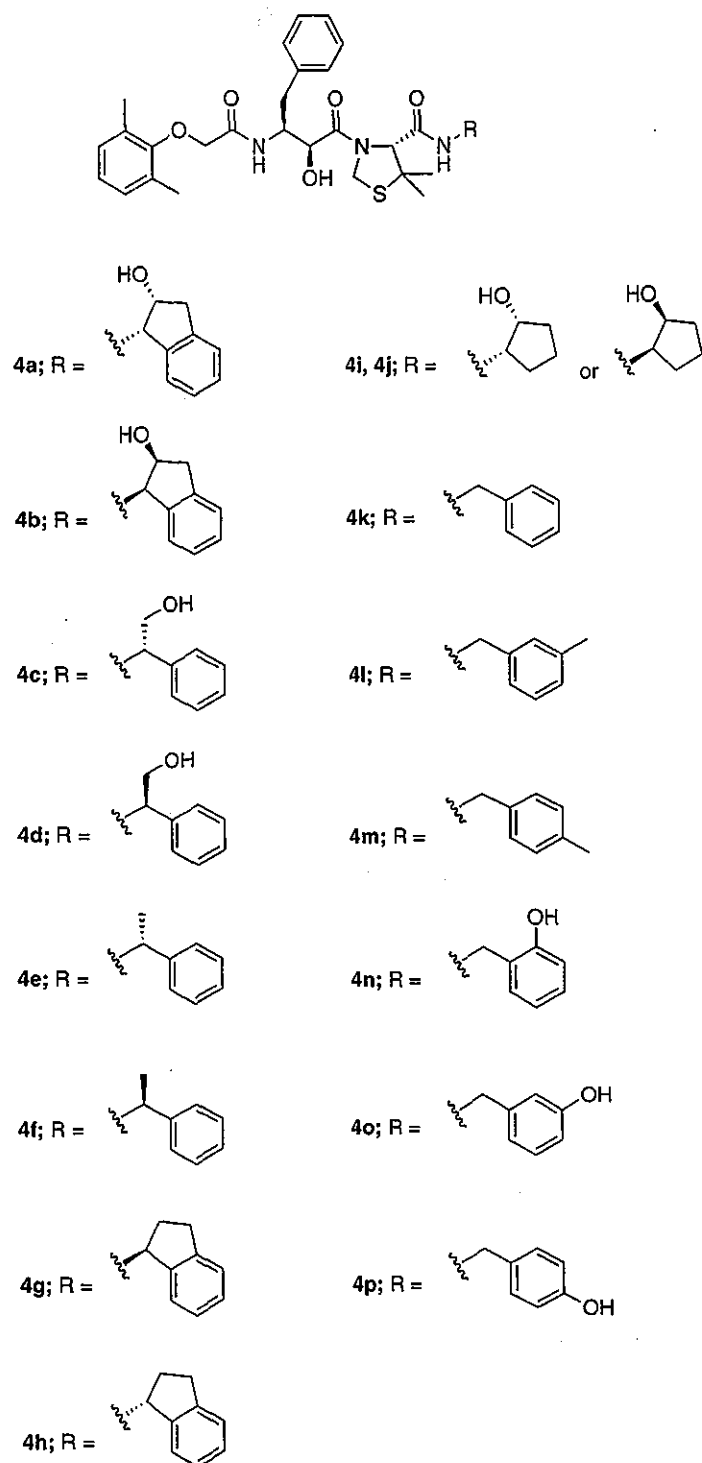
Inhibition constants (K_i) against Plm II and Cat D were obtained as described previously [5]. Briefly, the rate of substrate hydrolysis at 25 °C was measured using 400 nM protease in 10 mM sodium formate (pH 4.0), 163 µM chromogenic substrate [H-Ala-Leu-Glu-Arg-Thr-Phe-Phe(p-NO₂)-Ser-Phe-Pro-Thr-OH] which was purchased from California Peptide Research Inc., Napa, CA and 2%

DMSO with increasing amounts of inhibitor. K_i were estimated by fitting the data to standard equations for tight binding competitive inhibitors.

RESULTS AND DISCUSSION

As shown in Table 2, the previously reported compound **4a** [6], which has $(1S,2R)$-1-amino-2-indanol at the P_2' position, exhibited remarkably potent inhibitory activity with a K_i value of 0.5 nM. This activity is 140-fold more potent than KNI-727. On the other hand, compound **4b**, which has the opposite configuration of **4a**, exhibited a lower inhibitory activity, suggesting that the stereochemistry at the aminoindanol part of **4a** is favorable in the interaction with the S_2' pocket of Plm II. Compounds **4c** and **4d** have a phenylglycinol with a hydroxyl group, which is more flexible than that of aminoindanol at the P_2' position, but these compounds exhibited a lower inhibitory activity than **4a**. In order to understand the significance of the hydroxyl group and the aminoindanol structure in the potent enzyme inhibition, aminoindan was introduced at the P_2' position. The inhibitory activities of the resultant compounds **4g** and **4h** were relatively low. Aminocyclopentanol was also

Table 2 Inhibitory Activity Against Plm II, Cat D and HIV-1 PR

	Compound	Plm II K_i (nM)	Cat D K_i (nM)	HIV-1 PR % inhibition (50 nM)
4a	(KNI-10006)	0.5	2	98
4b	(KNI-10007)	71	806	21
4c	(KNI-10043)	22	53	16
4d	(KNI-10044)	532	1040	0
4e	(KNI-10030)	11	111	41
4f	(KNI-10063)	542	nd	85
4g	(KNI-10026)	15	105	97
4h	(KNI-1269)	99	nd	71
4i	(KNI-2017a)	108	nd	89
4j	(KNI-2017b)	709	nd	41
4k	(KNI-10046)	96	260	90
4l	(KNI-10053)	41	36	58
4m	(KNI-10052)	572	1321	55
4n	(KNI-10047)	55	252	77
4o	(KNI-10048)	99	771	89
4p	(KNI-10054)	273	1642	84

nd, not determined.

introduced at the P_2' position, in which the phenyl ring in **4a** was removed. However, compounds **4i** and **4j** showed a lower potency. From these results, it is suggested that the tight binding observed in the aminoindanol structure at the P_2' position is due to the effect of both the hydrophobic indan structure and its spatially arranged hydroxyl group which allows for proper interaction with Plm II.

The compounds (**4l–p**) with benzylamine derivatives exhibited lower inhibitory activities than KNI-840 which is introduced with o-methylbenzylamine. This result indicates that the inhibitory activity decreases in the order $o-$ > $m-$ > p-substitutions at the phenyl ring regardless of the presence of the methyl or hydroxyl group in these positions. In the case of the methyl substitution, a similar tendency was previously reported in HIV-1 PR inhibitors [17], suggesting that the recognition mechanism at the P_2' site of both Plm II and HIV-1 PR is similar.

In comparison with the Cat D inhibition, the selectivity of Plm II (K_i Cat D / K_i Plm II) did not improve in a series of modifications at the P_2' position (selectivity <11). Compound **4a** was also found to be a potent Cat D inhibitor (K_i = 2 nM). The replacement of t-butylamine in KNI-727 to aminoindanol in **4a** at the P_2' position contributed to a 650-fold increase in the Cat D inhibitory potency. This result suggests that a similar interaction induced by the hydroxyl group of aminoindanol enhances the binding affinity to Cat D, resulting in a decrease of the selectivity towards Plm II. This observed low selectivity, however, would not be a serious problem in future clinical use because the usual malaria treatment does not require a long time compared with the therapy of HIV infection.

As shown in Table 2, several compounds that exhibited potent Plm II inhibitory activity also exhibited potent inhibitory activities against HIV-1 PR. This is also attributed to the similarity in native substrate recognition between Plm II and HIV-1 PR. In particular, compound **4a** showed a high inhibitory activity against both Plm II and HIV-1 PR. This result suggests that the active conformations of **4a** in Plm II and HIV-1 PR may be similar. Therefore, a conformation of **4a** was obtained from the crystal structure of KNI-764 bound to HIV-1 PR (PDB entry, 2KZK) as a starting conformation. Then, its modeled structure bound to the active site of Plm II was generated adopting the conformation to the crystal structure of a complex of pepstatin A and Plm II (PDB entry, 1SME). The energy minimization with a MMFF94s force field was performed using the

Molecular Operating Environment modeling package (MOE 2003.02, Chemical Computing Group, Inc., Montreal, Canada). As shown in Figure 3, the indan moiety is fitted in the S_2' pocket of Plm II while the hydroxyl group of aminoindanol interacts with a hydroxyl group of Tyr192 by forming favorable hydrogen bonds. This suggests that the hydroxyl group of aminoindanol probably has the same role as the carbonyl group of Ser at the P_2' position in the substrate. A similar orientation of aminoindanol is shown in the case of indinavir in HIV-1 protease (PDB entry, 2BPX). However, the binding mode of aminoindanol in compound **4a** is different from the proposed model of a 1,2-dihydroxyethylene compound by Hallberg's group [12]. This is due to the fact that our modeled orientation of the phenyl group of compound **4a** is based on the active conformation of KNI-764 in HIV-1 PR.

Compound **4a** which exhibited the best activity against Plm II among all the test compounds also inhibited Plm I, IV and HAP [6]. This result suggests that compound **4a** might be able to inhibit the degradation of hemoglobin in the food vacuole. The activity of compound **4a** was also evaluated in *P. falciparum*-infected erythrocyte cultures. There was a significant reduction in the potency of compound **4a** in this assay (IC_{50} = 6.8 μM). This decrease can be explained by the fact that Plm inhibitors must penetrate four membranes (i.e. erythrocyte, parasitophorous vacuolar, parasite plasma, and food vacuole membranes) to reach their target. The excessive hydrophobicity of compound **4a** due to a dimethylphenoxyacetyl group at the P_2 position may cause a lowered cell penetration, hence suppressing its intrinsic activity.

In conclusion, novel Plm II inhibitors were designed and synthesized based on the substrate transition state. The Apns-Dmt scaffold was a Phe-Leu (P_1-P_1') transition-state mimic. Since native substrates contain a Ser residue at the P_2' position, a hydroxyl group was introduced with a fixed conformation and a bulky unit to fit the space of the S_2' pocket of Plm II. From the SAR at the P_2' position, (1S,2R)-1-amino-2-indanol was the best P_2' ligand (compound **4a**, KNI-10006) of the synthetic compounds, and both the hydroxyl group and the indan structure of the aminoindanol are important for its tight binding. Based on these observations, further modifications are under way to develop improved potential antimalarial compounds.
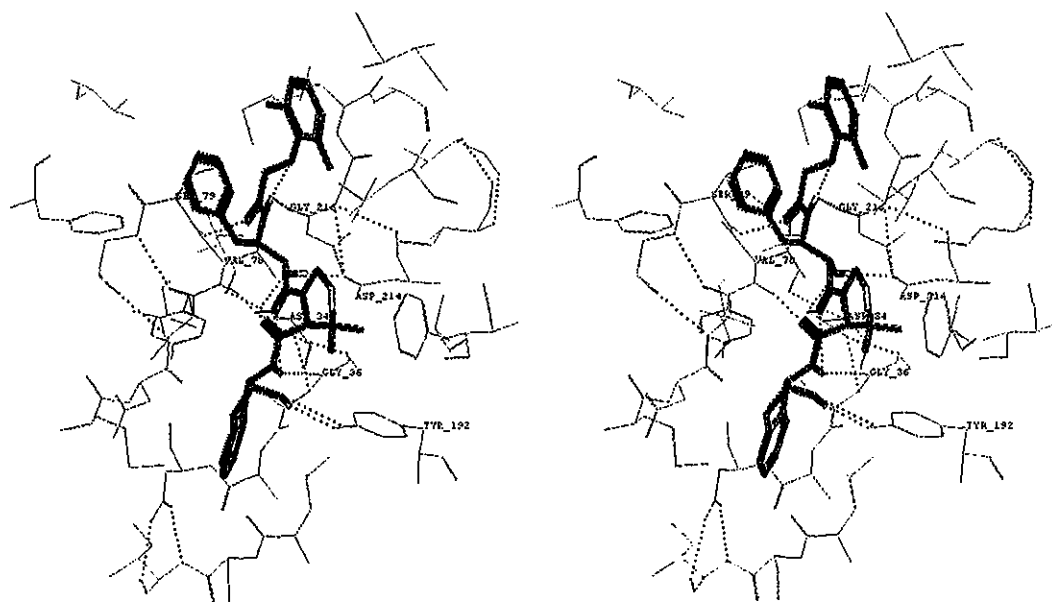
Acknowledgements

Figure 3 Modeled structure of compound **4a** bound to the active site of Plm II. Residues in the active site of Plm II are represented in light blue. Hydrogen bonds are represented with dotted line.

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20. KNI-10006 with Apns-Dmt scaffold is highlighted in Editors' choice. *Science* 2003; **301**: 143.

Minireview

Design of inhibitors against HIV, HTLV-I, and *Plasmodium falciparum* aspartic proteases

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Abstract

Aspartic proteases have emerged as targets for substrate-based inhibitor design due to their vital roles in the life cycles of the organisms that cause AIDS, malaria, leukemia, and other infectious diseases. Based on the concept of mimicking the substrate transition-state, we designed and synthesized a novel class of aspartic protease inhibitors containing the hydroxymethylcarbonyl (HMC) isostere. An unnatural amino acid, allophenylnorstatine [Apns; (2S,3S)-3-amino-2-hydroxy-4-phenylbutyric acid], was incorporated at the P1 site in a series of peptidomimetic compounds that mimic the natural substrates of the HIV, HTLV-I, and malarial aspartic proteases. From extensive structure-activity relationship studies, we were able to identify a series of highly potent peptidomimetic inhibitors of HIV protease. One highly potent inhibitor of the malarial aspartic protease (plasmepsin II) was identified. Finally, a promising lead compound against the HTLV-I protease was identified.

Keywords: AIDS; allophenylnorstatine; antiviral activity; HTLV-I; malaria; protease inhibitors.

Introduction

Aspartic proteases have become important targets of drug development for the treatment of many diseases due to the vital roles they play in the life cycles of many infectious organisms. The proposed mechanism of substrate cleavage by aspartic proteases (Figure 1) shows the formation of a transition state during amide hydrolysis. In designing aspartic protease inhibitors, we have followed the transition-state peptidomimetic principle, i.e., synthesis of a peptide substrate analog that contains a non-hydrolyzable transition-state isostere in place of the normal hydrolyzable P1–P1' amide bond. This strategy has been utilized to produce structurally diverse and

potent HIV protease inhibitors with different transition-state mimetics; indeed, several HIV protease inhibitors have already been approved for clinical treatment of AIDS patients (Kiso, 1996).

In our laboratory, we have successfully designed and synthesized a novel class of peptidomimetic aspartic protease inhibitors containing allophenylnorstatine [Apns; (2S,3S)-3-amino-2-hydroxy-4-phenylbutyric acid] with a hydroxymethylcarbonyl (HMC) isostere as therapeutic agents for a variety of diseases, such as AIDS, malaria, and leukemia (Kiso et al., 1999; Hamada and Kiso, 2003). Here, we briefly report our latest findings in this field.

HIV protease inhibitors

According to the United Nations program on HIV/AIDS (UNAIDS, 2003) data, the HIV virus is still spreading at an alarming rate; in 2003, approximately 5 million people contracted HIV, and many of these new cases were in sub-Saharan Africa and Southeast Asia. The high infection rate and the appearance of strains that are resistant to approved drugs have made finding novel drug candidates for AIDS treatment of the most challenging problems in medicinal chemistry. Since inhibition of the HIV-encoded protease results in the production of immature and non-infectious virions, intensive research has been devoted to the development of potent HIV protease inhibitors. This strategy has been validated by the approval of several HIV protease inhibitors for clinical use.

To design HIV protease inhibitors, we focused on the Phe-Pro scissile site of HIV protease substrates as the basis for the development of selective HIV protease inhibitors, because HIV protease can recognize Phe-Pro and Tyr-Pro sequences as cleavage sites (Table 1), while mammalian aspartic proteases cannot recognize these sites. Over the course of the last decade, we have reported a series of highly potent peptidomimetic HIV protease inhibitors containing Apns with a HMC isostere. Among these inhibitors, the tripeptide KNI-272 (Figure 2) showed extremely potent HIV protease inhibitory activity (K_i=5.5 pM), high antiviral activity against a wide spectrum of HIV strains, and low cytotoxicity (TC_{50}>80 μM). Furthermore, KNI-272 exhibited excellent enzyme selectivity, with practically no inhibition of other aspartic proteases, such as human plasma renin (IC_{50}>100 000 nM). NMR, X-ray crystallography, and molecular modeling studies have demonstrated that the HMC group in KNI-272 is able to interact excellently with the aspartic-acid carboxyl groups of the HIV-1 protease active site. The hydroxyl group in HMC forms a hydrogen bond to the
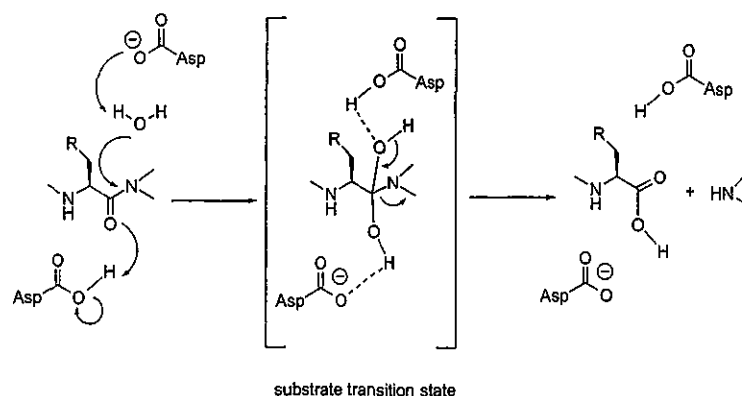
substrate transition state

Figure 1 Mechanism of peptide bond hydrolysis by aspartic proteases.

oxygen of Asp125 and the carbonyl oxygen forms a hydrogen bond to the protonated oxygen of Asp25 in essentially the same manner as the transition state of the substrate cleavage (Kiso et al., 1999; Abdel-Rahman et al., 2002).

Further structure-activity relationship (SAR) studies considering the subtle balance of lipophilicity-hydrophilicity and molecular size reduction (Mimoto et al., 1999) resulted in the generation of the conformationally constrained dipeptides KNI-727 and KNI-764 (Figure 2). While both compounds showed potent inhibitory activity against HIV protease, there is a large difference in antiviral activity. The EC_{50} value of KNI-727 was 1.0 μM against wild-type viruses, while that of KNI-764 was 82 nM. Furthermore, KNI-764 completely suppressed all HIV-1 and HIV-2 strains, as well as clinical HIV-1 variants that were highly resistant to all currently available protease inhibitors (Yoshimura et al., 1999).

Structural and thermodynamic information on KNI-764 showed that the better flexibility and adaptability of this inhibitor to the mutated HIV protease are the basis of the mechanism by which this inhibitor can minimize the effects of mutations conferring drug resistance (Velazquez-Campoy et al., 2001; Reiling et al., 2002; Vega et al., 2004). These results suggest that KNI-764 is a promising drug candidate for AIDS treatment, especially against resistant strains.

The structure-metabolism relationships of a series of peptidomimetic HIV protease inhibitors containing substituted Apns have recently been reported (Mimoto et al., 2004). In this study, these compounds not only showed higher stability against P2 phenol glucuronidation than KNI-764, but also showed more potent antiviral activity against both wild-type and multi-drug-resistant HIV-1.

Another important factor in the discovery of HIV protease inhibitors is their antiviral activity in the presence of human serum, as many potent HIV protease inhibitors show reduced antiviral activity in the presence of human serum. For this purpose, a SAR study of Apns-containing dipeptides was carried out, which led to the identification of KNI-1689 (Figure 2). The EC_{50} value of KNI-1689 was 10 nM against wild-type viruses in the absence of human serum, and the potency of this inhibitor was maintained in the presence of 50% human serum, with an EC_{50} value of 82 nM under these conditions (Kimura et al., 2004).

Plasmepsin II inhibitors

Malaria remains a major cause of serious illness and death. As the resistance of the parasite to conventional antimalarial drugs is increasing, the development of novel efficient therapies is urgently needed. Nearly all of the fatal cases of malaria are caused by *Plasmodium falci-*

Table 1 Substrate recognition sites for HIV-1 protease, plasmepsin II, and HTLV-I protease.

Enzyme	Cleavage site	Amino acid sequence						
		P4	P3	P2	P1–P1'	P2'	P3'	P4'
HIV-1	P17/p24	Gln	Arg	Gly	Tyr*Pro	Ile	Val	Gln
protease	P24/p1	Ala	Arg	Val	Leu*Ala	Glu	Ala	Met
	P1/p9	Ala	Thr	Ile	Met*Met	Gln	Arg	Gly
	P9/p6	Pro	Gly	Asn	Phe*Leu	Gln	Ser	Arg
	TF/PR	Ser	Phe	Asn	Phe*Pro	Gln	Ile	Thr
	PR/RT	Thr	Leu	Asn	Phe*Pro	Ile	Ser	Pro
	RT/RN	Ala	Glu	Thr	Phe*Tyr	Val	Asp	Gly
	RN/IN	Arg	Lys	Ile	Leu*Phe	Leu	Asp	Gly
Plasmepsin II	α 33/34	Glu	Arg	Met	Phe*Leu	Ser	Phe	Pro
	α 108/109	Leu	Leu	Val	Thr*Leu	Ala	Ala	His
	α 136/137	Ser	Thr	Val	Leu*Thr	Ser	Lys	Tyr
	β 32/33	Gly	Arg	Leu	Leu*Val	Val	Tyr	Pro
HTLV-I	p19/p24	Pro	Gln	Val	Leu*Pro	Val	Met	His
protease	p24/p15	Thr	Lys	Val	Leu*Val	Val	Gln	Pro
	TF1/PR	Ala	Ser	Ile	Leu*Pro	Val	Ile	Pro
	PR/p3	Pro	Val	Ile	Leu*Pro	Ile	Gln	Ala
	RT-RH/IN	Val	Leu	Gln	Leu*Ser	Pro	Ala	Phe

TF, transframe protein; PR, protease; RT, reverse transcriptase; RN, ribonuclease H; IN, integrase.
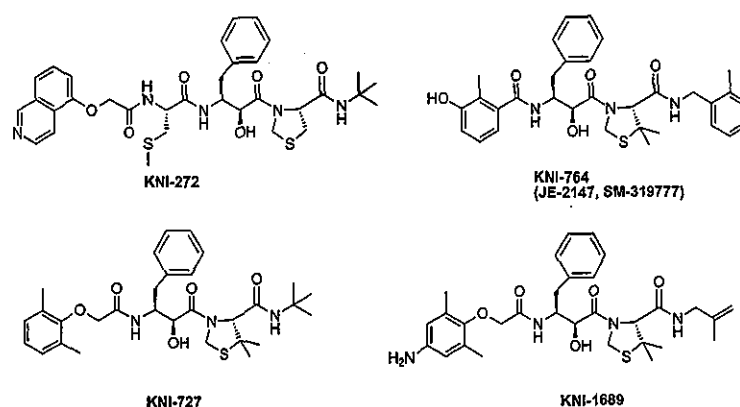
Figure 2 Apns-containing HIV protease inhibitors with the HMC isostere.

parum. This parasite encodes several proteases that are required for its hemoglobin degradation pathway. The acidic food vacuole of *P. falciparum* contains four aspartic proteases, plasmepsin (Plm) I, II, and IV, and a histo-aspartic protease (HAP), which are responsible for hemoglobin degradation (Coombs et al., 2001). These proteases have been recognized as promising targets for therapeutic intervention. Plm II can be easily produced by *E. coli* and the crystal structure of Plm II in complex with pepstatin has been solved. These findings made Plm II the most attractive target for inhibitor design (Boss et al., 2003). As shown in Table 1, the Plm II primary cleavage site is the peptide bond between Phe33 and Leu34 in the hemoglobin α-chain (Hamada and Kiso, 2003). Since the Phe-Leu structure is similar to the Apns-

containing scaffold, we hypothesized that our previously prepared HIV-1 inhibitors might also be effective inhibitors of Plm II. Therefore, we evaluated the Plm II inhibitory activity of selected Apns-containing HIV protease inhibitors (Nezami et al., 2002). As predicted, some of these compounds were able to inhibit Plm II potently. KNI-727 (Figure 2) showed potent inhibitory activity, with a K_i value of 70 nM, and 22-fold selectivity in comparison to the highly homologous human enzyme cathepsin D (Cat D). KNI-727 was also effective in killing the malaria parasite, with an EC_{50} value of 10 μM. Further SAR studies (Nezami et al., 2003; Kiso et al., 2004a,b) using KNI-727 as a lead compound resulted in a series of extremely potent inhibitors (Table 2). Among them, KNI-10006 was found to markedly inhibit Plm II, with a K_i value of 0.5 nM.

Table 2 Plasmepsin II and HIV-1 protease inhibition by Apns-based dipeptides.

Compound	P2′	K_i (nM)[a]		Inhibition[b] (%)
		Plm II	Cat D	
KNI-727		70	1300	96
KNI-840		20	80	98
KNI-10026		15	105	97
KNI-1269		99	-	71
KNI-10006		0.5	2	98
KNI-10007		71	806	21

[a]K_i values were determined as described in Nezami et al. (2002).
[b]Percentage HIV PR inhibition in the presence of 50 nM inhibitor.

P4   P3   P2   P1   P1'   P2' P3' P4'

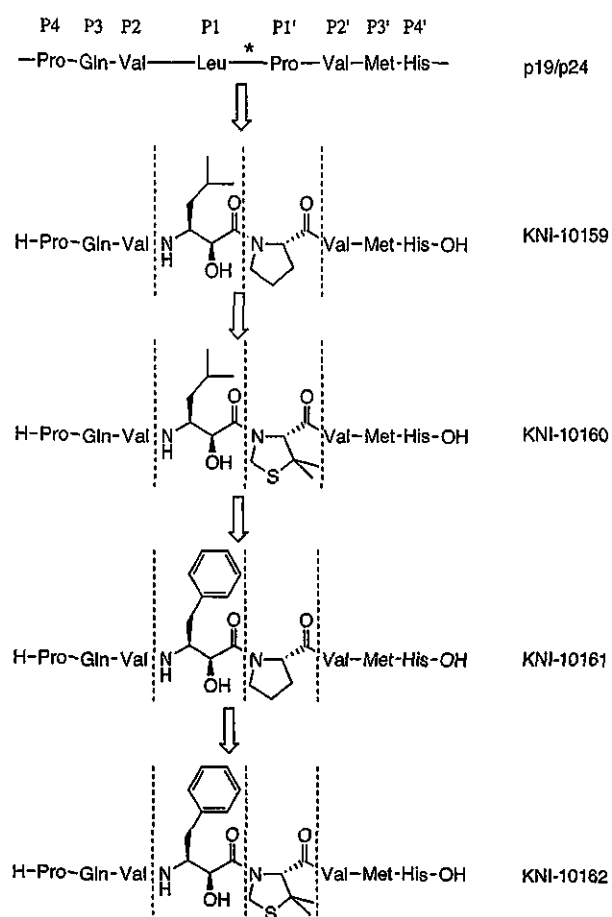—Pro—Gln—Val———Leu——*——Pro—Val—Met-His—   p19/p24

Figure 3   Design of substrate-based HTLV-1 protease inhibitors containing the HMC isostere.

KNI-10006 also showed potent inhibitory activity against Plm I and IV, and HAP.

HTLV-I protease inhibitors

The human T-cell leukemia virus type I (HTLV-I) is a retrovirus that is clinically associated with adult T-cell leukemia (ATL) and myelopathy/tropical spastic paraparesis (HAM/TSP) (Macchi et al., 2003). HTLV-I encodes a virus-specific aspartic protease that is responsible for processing the gag and gag-pro-pol polyproteins, and is required for proliferation of the retrovirus. Therefore, HTLV-I protease is a major target for the development of specific anti-HTLV-I agents (Shuker et al., 2003).

As we had already succeeded in developing HIV protease inhibitors based on the substrate transition-state mimetic concept using the HMC isostere, we thought that this concept might be successfully extended to the development of effective inhibitors of HTLV-I protease. To obtain compounds with potent HTLV-I protease inhibitory activity, we designed and synthesized new substrate-based HTLV-I protease inhibitors mimicking the p19/p24 sequences containing the Leu-Pro cleavage site shown in Table 1. We started the design process (Figure 3) by incorporating the unnatural amino acid allonorstatine [Anst;   (2S,3S)-3-amino-2-hydroxy-5-methylhexanoic acid] with the HMC isostere at the P1 site in an octapeptide, H-Pro-Gln-Val-Leu-Pro-Val-Met-His-OH. From

Table 3   HTLV-I protease inhibition by HMC-based compounds.

Compound	Protease inhibition (%)		
	HTLV-I		HIV[a]
	100 μM	5 μM	50 nM
KNI-10159	93	43	3
KNI-10160	100	63	37
KNI-10161	94	54	6
KNI-10162	100	66	58
Pepstatin A	-	17	23

For compound structures, see Figure 3.
[a]Percentage HIV PR inhibition in the presence of 50 nM inhibitor.

SAR studies of these compounds (Table 3), we were able to identify KNI-10162, which is an octapeptide similar to the p19/p24 sequence of the enzyme substrate in which the P1 and P1' amino acid residues are substituted with Apns and dimethylthioproline (Dmt), respectively. KNI-10162 showed 66% inhibition of the rec-HTLV-I protease at 5 μM (Maegawa et al., 2004). In addition, we observed that the recognition of these synthetic inhibitors by the HTLV-I protease was different from that of HIV-1 protease. These results suggest that KNI-10162 may be a useful lead compound for further modification in the development of specific anti-ATL and -HAM drugs (Maegawa et al., 2002).

Conclusion

In summary, we have designed and synthesized a promising class of peptidomimetic Apns-based aspartic protease inhibitors targeting infectious pathogens, such as HIV-1, P. falciparum, and HTLV-I. The design of these inhibitors is based on the substrate transition-state mimetic concept. From SAR studies of the HIV-1 protease inhibitors, we were able to identify highly potent compounds that have strong antiviral activity against both wild-type and drug-resistant viruses. KNI-10006 proved to be the most potent inhibitor not only against Plm II, but also against Plm I and IV, and HAP produced by P. falciparum. Finally, in a search for inhibitors of HTLV-I protease, KNI-10162 was identified as a promising lead compound for further SAR studies.

Acknowledgments

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