Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 366–370
Bioorganic & Medicinal Chemistry Letters

Truncation and non-natural amino acid substitution studies on HTLV-I protease hexapeptidic inhibitors

Jeffrey-Tri Nguyen,[a] Meihui Zhang,[a,b] Henri-Obadja Kumada,[a] Ayako Itami,[a] Keiji Nishiyama,[a] Tooru Kimura,[a] Maosheng Cheng,[b] Yoshio Hayashi[a,c] and Yoshiaki Kiso[a,*]

[a]*Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science and 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan*
[b]*Key Laboratory of New Drugs Design and Discovery of Liaoning Province, School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang 110016, PR China*
[c]*Department of Medicinal Chemistry, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan*

Received 26 September 2007; revised 16 October 2007; accepted 18 October 2007
Available online 24 October 2007

Abstract—The culprit behind adult T-cell leukemia, myelopathy/tropical paraparesis, and a plethora of inflammatory diseases is the human T-cell leukemia virus type 1 (HTLV-I). We recently unveiled a potent hexapeptidic HTLV-I protease inhibitor, KNI-10166, composed mostly of natural amino acid residues. Herein, we report the derivation of potent tetrapeptidic inhibitor KNI-10516, possessing only non-natural amino acid residues.

Adult T-cell leukemia (ATL), an aggressive malignancy of CD4 T lymphocytes, that presents as skin lesions, lymphadenopathy, and hepatosplenomegaly, was first described by Takatsuki and co-workers in Japan in 1977.[1] HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), predominantly associated with CD8 T lymphocytes in the later stage of the disease, primarily affects the spinal cord at the thoracic level causing symmetrical weakness and paralysis of the lower limbs.[2] The causative agent for ATL, HAM/TSP, and several inflammatory diseases was identified in 1980 by Gallo and co-workers as the human T-cell leukemia virus type 1 (HTLV-I).[2,3] Infecting 15–20 million people worldwide, the virus is often transmitted through breast-feeding, sexual intercourse, blood transfusion, and the sharing of contaminated injection devices, thereby allowing the virus to be endemic in the equatorial regions of Africa, Central and South America, the Caribbean, Melanesia, the Middle East, and south-western Japan, and spreading to the United States and Europe through injection drug use and sexual transmission.[2,4] Although there are therapies to alleviate the symptoms of the diseases, there is no effective treatment to eradicate HTLV-I. Infection with HTLV-I is lifelong. HTLV-I protease (PR), first identified and isolated in 1989, plays a pivotal role in HTLV-I replication.[5] Inhibition of HTLV-I PR would essentially stop viral replication and provide a possible cure to HTLV-I related diseases, thereby directly attacking at the source of the problems. The genome for HTLV-I encodes for several key proteins, namely Gag, Pro, Pol, and Env.[6] HTLV-I PR cleaves the Gag and Pol precursor polyproteins into several shorter proteins that are subsequently assembled and developed into a mature virion that ultimately becomes the cause for HTLV-I-associated diseases. Hence, our approach to the problem is to inhibit HTLV-I PR with a substrate mimic, and thereby stopping the spread of the virus.

One of the cleavage sites involved in the processing of the precursor Gag polyprotein, the matrix/capsid cleavage site, can accommodate for a specific substrate (**1**, Table 1).[7] In our previous work, based on the substrate sequence (**1**) and the concept of ''transition-state mimic'' at the P_1/P'_1 cleavage position using (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid (allophenylnorstatine,

Keywords: Human T-cell leukemia virus; Adult T-cell leukemia; Human immunodeficiency virus; Protease inhibitor.
* Corresponding author. Tel.: +81 75 595 4635; fax: +81 75 591 9900; e-mail: kiso@mb.kyoto-phu.ac.jp

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.10.066

Table 1. HTLV-I PR substrates and peptidic inhibitors

Compounds (KNI-No.)		Structure										HTLV-I PR IC_{50}[a] (nM)	HIV-1 PR inhibition[b] (%)
			P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	P'_5		
1	Substrate	H	Pro	Gln	Val	Leu	Pro	Val	Met	His	Pro	OH	—	—
2	10161	H	Pro	Gln	Val	Apns	Pro	Val	Met	His	OH		159	<30
3	10162	H	Pro	Gln	Val	Apns	Dmt	Val	Met	His	OH		152	58
4	10127		Ac	Gln	Val	Apns	Dmt	Val	Met	NH_2			353	96
5	10166		Ac	Ile	Ile	Apns	Dmt	Ile	Met	NH_2			88	98

[a] HTLV-I PR inhibition, IC_{50} (nM), using HTLV-I PR mutant L40I.
[b] HIV-1 PR inhibition (%) at 50 nM of the test compound.

Apns) having a hydroxymethylcarbonyl (HMC) isostere, we developed potent octapeptidic HTLV-I PR inhibitor KNI-10161 (**2**).[8] KNI-10162 (**3**), which differed by a P'_1 non-natural proline isostere, namely (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (Dmt), exhibited equipotency. The result of a truncation study led to a smaller hexapeptidic inhibitor, KNI-10127 (**4**), with a fair loss of activity.[9] In order to recover inhibitory activity, we performed substitution studies by building small amino acid libraries at each residue position to derive compound KNI-10166 (**5**). Considering that the in vivo efficacy of inhibitors is severely compromised by their susceptibility to proteolytic degradation and difficulty in penetrating cells, we felt a need to explore non-natural amino acid residues and further reduce the size of the inhibitor. Herein, we report the design, synthesis, and activity of several novel hexa-, penta-, and tetrapeptidic HTLV-I PR inhibitors possessing non-natural amino acid residues.

We performed a truncation study on KNI-10166 (**5**, Table 2). Compounds **6** and **7** were synthesized and their inhibitory activities against HTLV-I and human immunodeficiency virus type 1 (HIV-1) PRs were determined using inhibitor **5** as a comparative reference. Compound **6**, which lacked a P_3 Ile moiety at the N-terminal, had a drastic loss of inhibitory activity against HTLV-I PR.[10] Compound **7**, which lacked a P'_3 Met moiety at the C-terminal, retained some activity against HTLV-I PR, thereby suggesting that the P_3 residue is a higher determinant of activity than the P'_3 residue.

In our preceding work, we built small libraries with natural amino acids at each residue position to derive inhibitor **5** and noted that the S'_2, S_2, and S_3 sites best accommodated for Ile residues.[9] To avoid recognition by endopeptidases that could prematurely metabolize the compound in the body, in the current study, we opted for non-natural amino acid residue substitution and chose L-*tert*-leucine (Tle) and L-(+)-α-phenylglycine (Phg) to mimic the branched amino acid, Ile (Table 3). Among the Tle and Phg residues, the S_3 site accommodated better Phg (cf. **8** and **9**), whereas a P_2 Tle would exhibit more potent HTLV-I PR inhibitory activity (cf. **10** and **11**), and Phg is the preferred moiety at the P'_2 position (cf. **13** and **14**).

Although in our published work on HTLV-I PR inhibitors,[8,9] we designed inhibitors possessing an Apns residue with an HMC isostere at the P_1 position, based on our published HIV-1 PR inhibitor studies, in the current study, we explored Apns' diastereomer (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid (phenylnorstatine, Pns) and did not observe an improvement in HTLV-I PR inhibition (cf. **5** and **12**).

We noted that a P'_3 Ala residue could be accommodated by the reverse transcriptase–ribonuclease H/integrase S'_3 cleavage junction,[6] and synthesized compound **15** that exhibited lower PR inhibitory activity than reference inhibitor **5**. In our previous study, we discovered that KNI-10153 and KNI-10156, each, respectively, possessing a P'_3 Phe or Gln residue, exhibited fairly potent HTLV-I PR inhibition,[9] and accordingly, we synthesized and evaluated HTLV-I PR inhibitory activity of compounds **16** and **17**, in which a corresponding P'_3 Phe or Gln residue was present. Compounds **5** and **16** exhibited similar potencies, while compounds **15** and **17** were equipotent.

From the results of the truncation study on compound **5** (Table 2), we decided to optimize the P_3–P'_1 residues and then remove the P'_2–P'_3 residues. First, we synthesized P_3–P'_1 optimized inhibitor **18**, which exhibited potent inhibitory activity against both HTLV-I and HIV-1 PRs (cf. **5**). In consideration of the vast structural differences between the P'_3 residues in compounds **5** and **15–17**, and that they exhibited minor variations in HTLV-I PR inhibitory activity (cf. **5** and **16**; **15** and **17**), we inferred that the P'_3 residue is a minor determi-

Table 2. Truncation study on KNI-10166

Compounds (KNI-No.)		Structure								HTLV-I PR IC_{50}[a] (nM)	HIV-1 PR inhibition[b] (%)
			P_3	P_2	P_1	P'_1	P'_2	P'_3			
6	10167		Ac	Ile	Apns	Dmt	Ile	Met	NH_2	>1000	89
5	10166	Ac	Ile	Ile	Apns	Dmt	Ile	Met	NH_2	88	98
7	10168	Ac	Ile	Ile	Apns	Dmt	Ile	NH_2		249	57

Shaded value denotes that a truncation study has been performed.
[a] HTLV-I PR inhibition, IC_{50} (nM), using HTLV-I PR mutant L40I.
[b] HIV-1 PR inhibition (%) at 50 nM of the test compound.

Table 3. Small libraries with non-natural amino acids from KNI-10166

Compounds (KNI-No.)		Structure								HTLV-I PR IC_{50}[a] (nM)	HIV-1 PR inhibition[b] (%)
			P_3	P_2	P_1	P'_1	P'_2	P'_3			
8	10198	Ac	Tle	Ile	Apns	Dmt	Ile	Met	NH_2	201	98
9	10199	Ac	Phg	Ile	Apns	Dmt	Ile	Met	NH_2	102	98
10	10194	Ac	Ile	Tle	Apns	Dmt	Ile	Met	NH_2	145	97
11	10195	Ac	Ile	Phg	Apns	Dmt	Ile	Met	NH_2	320	88
12	10188	Ac	Ile	Ile	Pns	Dmt	Ile	Met	NH_2	180	73
13	10196	Ac	Ile	Ile	Apns	Dmt	Tle	Met	NH_2	180	79
14	10197	Ac	Ile	Ile	Apns	Dmt	Phg	Met	NH_2	123	86
15	10193	Ac	Ile	Ile	Apns	Dmt	Ile	Ala	NH_2	160	82
16	10192	Ac	Ile	Ile	Apns	Dmt	Ile	Phe	NH_2	95	98
17	10191	Ac	Ile	Ile	Apns	Dmt	Ile	Gln	NH_2	159	93
18	10220	Ac	Phg	Tle	Apns	Dmt	Ile	Met	NH_2	101	99
19	10221	Ac	Phg	Tle	Apns	Dmt	Ile	Gln	NH_2	84	98
20	10247	Ac	Phg	Tle	Apns	Dmt	Ile	NH_2		144	93

[a] HTLV-I PR inhibition, IC50 (nM), using HTLV-I PR mutant L40I.
[b] HIV-1 PR inhibition (%) at 50 nM of the test compound.

nant of activity. To confirm, we synthesized another potent inhibitor (**19**) which possessed a P'_3 Gln residue (cf. **5**, **17**, and **18**). Compound **20**, which is a P'_3-less analogue of inhibitors **18** and **19**, exhibited moderate inhibitory activity against HTLV-I PR and higher potency than inhibitor **7**, a P'_3-less analogue of compound **5**. Further truncation of inhibitor **20** at either the P_3 residue or P'_2 residue nearly abolished inhibitory activity against HTLV-I PR.[10]

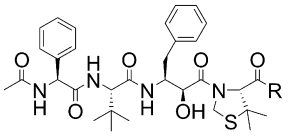
To replace the remaining natural amino acid in compound **20**, we selected the same C-terminal capping moiety used by Akaji et al.[11] However, the resulting compound (**21**, Table 4) exhibited low activity against HTLV-I PR. Optimizing the substituents on the benzyl ring, we derived potent inhibitor **22**, although other analogues exhibited low to moderate inhibitory activity (unpublished). Approaching the problem from a different perspective, we decided to probe the S'_2 pocket by increasing the bulk near the β-carbon of the capping group in compounds **23–28**. Surprisingly, most compounds exhibited moderate to high potency against HTLV-I PR with what seems to be a general trend of improved potency proportional with increased bulk, resulting in compound **27** being the more effective inhibitor. Of interest, the P'_1 capping moieties in compounds **21** and **22** are structurally similar to Phg (cf. **14**), whereas the P'_1 capping moiety in compound **28** resembles Tle (cf. **13**).

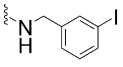
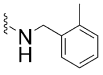
Comparative computer-assisted docking experiments with HTLV-I PR performed on the reference compound, KNI-10166 (**5**),[9] and title compound, KNI-10516 (**27**), revealed improved van der Waals interactions between the P_2 and P_3 side chains of compound **27**, and the respective pockets (not shown). Similar hydrogen bond interactions were present in both compounds **5** and **27**, with the exception of interactions near the non-existent P'_2–P'_3 residues in compound **27** (Fig. 1).

HTLV-I and HIV-1 are distant cousins from the *Retroviridae* (retrovirus) family. In our previous study, we observed that hexapeptidic HTLV-I PR inhibitors often exhibit potent HIV-1 PR inhibitory activity, and not necessarily vice versa.[9] The current study suggests that the trend also applies for shorter inhibitors.

The synthesis of compounds **6–20** was achieved by Fmoc-based solid peptide synthesis on Rink amide AM resin using previously described general techniques.[9] The respective C-terminal Fmoc-amino acid was loaded onto a Rink amide AM resin by DIPCDI-

Table 4. P'_2 residue modification on KNI-10247



Compounds (KNI-No.)		R	HTLV-I PR IC_{50}[a] (nM)	HIV-1 PR inhibition[b] (%)
21	10401		473	97
22	10252		103	99
23	10496		131	>99
24	10502		193	98
25	10592		146	99
26	10455		140	>99
27	10516		107	>99
28	10600		113	99

[a] HTLV-I PR inhibition, IC_{50} (nM), using HTLV-I PR mutant L40I.
[b] HIV-1 PR inhibition (%) at 50 nM of the test compound.

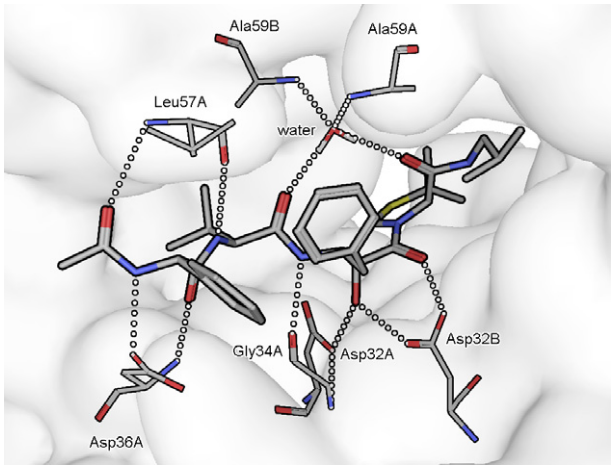


Figure 1. Computer model of KNI-10516 (**27**) in the active site of HTLV-I PR. Dotted lines represent possible hydrogen bond interactions throughout the backbone of the inhibitor and HTLV-I PR's Asp32A, Gly34A, Asp36A, Leu57A, Ala59A, Asp32B, and Ala59B. Interactions between Ala59A and Ala59B in the hairpin regions of HTLV-I PRs flaps and the inhibitor is mediated by a water molecule. The inhibitor's transition-state mimic HMC isostere interacts with catalytic Asp32A and Asp32B.

HOBt (1,3-diisopropylcarbodiimide; 1-hydroxybenzotriazole) method. Piperidine (20%) in DMF was employed to remove the Fmoc protection group, while coupling with an appropriate Fmoc-protected amino acid was performed using the DIPCDI-HOBt method for each chain-elongation step. N-Acetylation was performed with acetic anhydride and Et_3N in DMF. Cleavage from the resin was performed with trifluoroacetic acid, *m*-cresol, thioanisole, and water. Compounds **21–28** were synthesized by standard solution phase peptide synthesis in which sequential coupling of a respective amine to a corresponding Boc-protected amino acid was performed in DMF with EDC·HCl-HOBt (1-ethyl-3-(3-dimethylaminopropyl)carboiimide hydrochloride) or BOP (benzotriazol-1-yl-oxy-tris-dimethylaminophosphonium hexafluorophosphate) as coupling reagents in the presence of Et_3N. Coupling with BOP was often more efficient than with EDC·HCl-HOBt. Removal of the Boc protection group was achieved with 4 N HCl in dioxane during each chain-elongation step. Similarly, P_3 N-acetylation was performed with acetic acid, BOP, and Et_3N in DMF. After preparative HPLC purification, all target compounds (**6–28**) were >98% pure by analytical HPLC. The identities of the compounds were confirmed by ESI-Q MS and/or TOF MS. To improve the reliability of our HTLV-I PR assay, we developed a more proteolytic HTLV-I PR L40I mutant[12] against a substrate, which consequently dramatically reduced incubation time from 6 h to 30 min when compared to the former method.[8] This more efficient mutant permitted a cost-effective means of determining IC_{50} values.[13] Recombinant HIV-1 PR assay[8] and computer-assisted docking experiments[9] were performed in similar manners as previously described.

In the current study, rational drug design was applied to the potent lead hexapeptidic inhibitor, KNI-10166 (**5**, IC_{50} = 88 nM). A substitution study with Tle and Phg at the respective P_2 and P_3 positions, followed by removal of the P'_2–P'_3 residue and optimization of the P'_1 cap moiety from the structural design of KNI-10166, led to the intuitive discovery of KNI-10516 (**27**), which is a tetrapeptidic HTLV-I PR inhibitor, composing of only non-natural amino acid residues and exhibiting highly potent inhibition (IC_{50} = 107 nM) against HTLV-I PR.

Acknowledgements

This research was supported by The Frontier Research Program; The 21st Century COE Program from The Ministry of Education, Culture, Sports, Science and Technology, Japan; and Japan Society for the Promotion of Science's Post-Doctoral Fellowship for Foreign Researchers. We are grateful to Mr. T. Hamada for mass spectra and HIV-1 PR assay determinations.

References and notes

1. Takatsuki, K. *Retrovirology* **2005**, *2*, 16.
2. Jacobson, S. *J. Infect. Dis.* **2002**, *186*(Suppl. 2), S187.
3. Gallo, C. *Retrovirology* **2005**, *2*, 17.
4. Proietti, F. A.; Carneiro-Proietti, A. B. F.; Catalan-Soares, B. C.; Murphy, E. L. *Retrovirology* **2005**, *24*, 6058.
5. Hatanaka, M.; Nam, S. H. *J. Cell. Biochem.* **1989**, *40*, 15.
6. Shuker, S. B.; Mariani, V. L.; Herger, B. E.; Dennison, K. J. *Chem. Biol.* **2003**, *10*, 373.
7. Ha, J. J.; Gaul, D. A.; Mariani, V. L.; Ding, Y. S.; Ikeda, R. A.; Shuker, S. B. *Bioorg. Chem.* **2002**, *30*, 138.
8. Maegawa, H.; Kimura, T.; Arii, Y.; Matsui, Y.; Kasai, S.; Hayashi, Y.; Kiso, Y. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5925.
9. Kimura, T.; Nguyen, J.-T.; Maegawa, H.; Nishiyama, K.; Arii, Y.; Matsui, Y.; Hayashi, Y.; Kiso, Y. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3276.
10. KNI-10167 (**6**), Ac-Ile-Apns-Dmt-Ile-Met-NH_2; 39% HTLV-I PR inhibition at 1 μM; 89% HIV-1 PR inhibition at 50 nM. KNI-10472, Ac-Tle-Apns-Dmt-Ile-NH_2; 5% HTLV-I PR inhibition at 600 nM; 30% HIV-1 PR inhibition at 50 nM. KNI-10471, Ac-Phg-Tle-Apns-Dmt-NH_2; 8% HTLV-I PR inhibition at 600 nM; 36% HIV-1 PR inhibition at 50 nM.
11. Naka, H.; Teruya, K.; Bang, J. K.; Aimoto, S.; Tatsumi, T.; Konno, H.; Nosaka, K.; Akaji, K. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3761.
12. A synthesized HTLV-I PR gene was introduced into *Escherichia coli* BL21(DE3)-pLysS cells as described in Ref. 8. The gene was extracted and mutated to construct a plasmid as follows: (1) an N*de*I restriction site was added to the 5′ end; (2) the Leu40 codon was mutated to an Ile codon to block autolysis; and (3) a stop codon and a P*st*I restriction site were introduced 3′ to the Leu125 codon. The gene was cloned into pColdI (Takara-Bio) by using the N*de*I and P*st*I restriction sites to afford pColdI/HTLV-I PR, and introduced into *E. coli* BL21 cells. Expression of the gene and ensuing procedures were performed as described in Ref. 8.
13. The HTLV-I PR inhibitory activity was determined by measuring the rate of hydrolysis of APQVL(*p*-nitrophenylalanine)VMHPL. The enzyme reaction mixture (50 μL) contained 1 μg protease ($OD_{595} \approx 0.050$, as a dimer),

0.2 mM substrate, 0.2 M citrate buffer (pH 5.3), 1 mM dithiothreitol, 1 M NaCl, 5 mM EDTA, 6% v/v glycerol, and 2% v/v DMSO solution at different inhibitor concentrations, and the reaction proceeded at 37 °C for 30 min, then it was terminated with 20% aqueous trichloroacetic acid (15 μL). The hydrolyzed substrate fragments and/or non-hydrolyzed substrate were quantified by HPLC using a YMC-Pack Pro C18 column (linear gradient: 5–35% CH_3CN in 0.1% aqueous TFA; 1.0 mL/min; 215 nm; 40 °C; calculated from standard curves of the areas under the peaks at 6, 10, and 13 min). IC_{50} value was calculated using a probit model.