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Chipping at large, potent human T-cell leukemia virus type 1 protease inhibitors to uncover smaller, equipotent inhibitors

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Abstract—The human T-cell leukemia virus type 1 (HTLV-I) causes adult T-cell leukemia and several severe chronic diseases. HTLV-I protease (PR) inhibition stops the propagation of the virus. Herein, truncation studies were performed on potent octapeptidic HTLV-I PR inhibitor KNI-10161 to derive small hexapeptide KNI-10127 with some loss in activity. After performing residue-substitution studies on compound KNI-10127, HTLV-I PR inhibitory activity was recovered in inhibitor KNI-10166.

First identified in the early 1980s, the human T-cell leukemia virus type 1 (HTLV-I) infects T-cells causing malignant proliferation of adult T-cell leukemia (ATL) that infiltrates skin and brain leading to various chronic diseases including uveitis, arthritis, infective dermatitis, as well as HTLV-I associated myelopathy/tropical spastic paraparesis (HAM/TSP) in which patients present with a gradual onset of symmetrical weakness, upper motor neuronal signs, mainly affecting lower limbs.[1,2] Infection with the oncogenic retrovirus is endemic in south-western Japan, the Caribbean Basin, South America, Central and West Africa, the Middle East, and the Pacific region where, for example, 15–25% individuals in Japan, infected mainly via breast milk transmission, are viral carriers out of which up to 6% will succumb to the disease.[3] At the present time, there is no effective curative treatment for ATL and HTLV-I infection.

The HTLV-I protease (PR), first identified and isolated in 1989, plays a key role in the replication of HTLV-I.[4] The genome for HTLV-I is flanked by two long terminal repeats with the following encoded gene sequences: Gag, Pro, Pol, Env, and regulatory proteins.[2] HTLV-I PR cleaves the 55 kDa Gag precursor polyprotein into matrix (MA), capsid (CA) and nucleocapsid (NC) proteins; and the 95 kDa Pol precursor polyprotein into reverse transcriptase-ribonuclease H (RT-RH) and integrase (IN).[5] These proteins are assembled and developed into a mature virion leading to the pathogenesis of ATL and HAM/TSP. Without any doubt, the development of HTLV-I PR inhibitors offers an attractive solution to the currently incurable disease, because inhibition of the processing of the Gag, Gag-Pro, and Gag-Pol polyproteins would essentially stop viral replication.

In our recent study, we designed and synthesized a potent HTLV-I PR inhibitor, KNI-10161 (**7**), based on a peptide substrate that could be accommodated by the MA/CA cleavage site (Tables 1 and 2).[6] KNI-10161 was designed with a (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid (allophenylnorstatine, Apns) moiety at the P_1 position having a hydroxymethylcarbonyl (HMC) isostere transition-state mimic. In the work described herein, we explored peptide chain length and residue type requirements for HTLV-I PR inhibition.

The process of optimizing an already potent peptidic inhibitor down to a smaller inhibitor usually entails some loss of potency. However, smaller inhibitors often offer several potential benefits including improved body distribution, cell penetration, and administration vehicle dissolution. Moreover, the sacrificed potency could be recovered or even surpassed when each residue in the

Abbreviations: Apns, (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid, allophenylnorstatine; Dmt, (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid; Mta, (*R*)-*S*-methyl-L-cysteine, L-methylthioalanine; DP-CDI, 1,3-diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole.

Keywords: Human T-cell leukemia virus; Adult T-cell leukemia; Human immunodeficiency virus; Protease inhibitor.

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Table 1. Residues accommodated at different HTLV-I PR cleavage sites[a]

Cleavage site		P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	
MA/CA	...	Pro	Gln	Val	Leu	Pro	Val	Met	His	...
CA/NC	...	Thr	Lys	Val	Leu	Val	Val	Gln	Pro	...
Gag/PR	...	Ala	Ser	Ile	Leu	Pro	Val	Ile	Pro	...
PR/Pol	...	Pro	Val	Ile	Leu	Pro	Ile	Gln	Ala	...
Pro/RT	...	Pro	Ala	Val	Leu	Gly	Leu	Glu	Leu	...
RT-RH/IN	...	Val	Leu	Gln	Leu	Ser	Pro	Ala	Asp	...

[a] Modified from Shuker et al.[5]

Table 2. HTLV-I PR inhibitory activity for inhibitors with different number of residues

Compounds (KNI-No.)		Structure									HTLV-I inhibition (%)[a]	HIV-1 inhibition (%)[b]
			P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4		
1	10112				H	Apns	Pro	Val	Met	His	OH	<30	<30
2	10102				Ac	Apns	Pro	Val	Met	His	OH	<30	<30
3	10103			H	Val	Apns	Pro	Val	Met	His	OH	<30	<30
4	10104			Ac	Val	Apns	Pro	Val	Met	His	OH	<30	<30
5	10105		H	Gln	Val	Apns	Pro	Val	Met	His	OH	<30	<30
6	10108		Ac	Gln	Val	Apns	Pro	Val	Met	His	OH	49	<30
7	10161	H	Pro	Gln	Val	Apns	Pro	Val	Met	His	OH	94	<30
8	10109	H	Pro	Gln	Val	Apns	Pro	Val	Met	NH_2		86	<30
9	10110	H	Pro	Gln	Val	Apns	Pro	Val	NH_2			<30	<30
10	10116	H	Pro	Gln	Val	Apns	Pro	NH_2				<30	<30
11	10162	H	Pro	Gln	Val	Apns	Dmt	Val	Met	His	OH	>99	58
12	10127		Ac	Gln	Val	Apns	Dmt	Val	Met	NH_2		66	96

[a] HTLV-I PR inhibition (%) at 100 μM of the test compound.
[b] HIV-1 PR inhibition (%) at 50 nM of the test compound.

inhibitor is optimized for HTLV-I PR inhibitory activity.

Our first endeavor was to examine the influence of the number of residues on HTLV-I PR inhibitory activity using our recently reported inhibitor KNI-10161 (**7**) as reference (Table 2).[6] In the current study, we methodically 'removed' the C- and N-terminal residues of compound **7** to determine the critical points at which inhibitory activity is nearly absent (<30% inhibition at 100 μM of the test compound). Several shorter inhibitors (**1**–**6**) with either a free or acetylated N-terminal amine in the non-prime regions were synthesized and their respective HTLV-I PR inhibitory activities were determined. Compounds **1**–**4** exhibited low potencies against HTLV-I PR. The reduced HTLV-I PR activity in compounds **5** and **6** relative to inhibitor **7** is in agreement with observations made by Tözsér et al. that the removal of the P_4 amino acid residue from a peptidic substrate resulted in a decrease in catalytic efficiency.[7] The presence of some inhibitory activity in compound **6** possessing a relatively smaller P_4 acetyl moiety than compound **7** with a P_4 Pro suggests that the PR could accommodate for shorter inhibitors. Inhibitors with shorter number of prime residues (**8**–**10**) were also synthesized to evaluate structure–activity relationships. Compound **9** and **10**'s inhibitory activities were low. The result for compound **8**, in which P'_4 His is absent and P'_3 Met's carboxylic acid has been altered to an amide, indicates that the presence of a P'_4 residue is a minor determinant for inhibitory activity. Interestingly, another research group studying HTLV-I PR inhibitors based on the PR/Pol cleavage site (Table 1) arrived at a similar conclusion that a seven-residue peptide is required for substrate recognition by HTLV-I PR.[8] Recently, Li et al. reported the X-ray crystallography structure of an inhibitor in complex with HTLV-I PR which revealed that the P_4 and P'_4 residues are near to the outside of the active site, and thus, their contributions to activity are less significant.[9]

Considering that P_3-to-P'_4 heptapeptide **6** and P_4-to-P'_3 heptapeptide **8** both retained some HTLV-I PR inhibitory activity, our study urged us to explore smaller inhibitors consisting of six residues spanning from P_3 to P'_3. Compound **12** was synthesized while keeping in mind that our previous study revealed that a more conformationally constrained and bulkier P'_1 (*R*)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (Dmt) residue exhibited slightly more potent inhibitory activity than a P'_1 Pro residue (cf. compounds **7** and **11**).[6] In an attempt to minimize the inhibitor's size, HTLV-I PR inhibitory activity was greatly reduced from >99% (**11**) to 66% (**12**).

Our second endeavor was to optimize each residue in reference compound **12** for inhibitory activity by building small libraries of amino acids at each residue position (Table 3). We began with the P_3 residue position while considering increasing lipophilicity so as to ameliorate cell penetration in future cell-based assays. Acknowledging that the PR could cleave different substrates, compounds **13**–**15** were synthesized based on the P_3 residue of the respective PR/Pol, Pro/RT, and

Table 3. HTLV-I PR inhibitory activity for hexapeptidic inhibitors

Compounds (KNI-No.)		Structure								HTLV-I inhibition (%)[a]	HIV-1 inhibition (%)[b]
			P_3	P_2	P_1	P'_1	P'_2	P'_3			
12	10127	Ac	Gln	Val	Apns	Dmt	Val	Met	NH_2	66	96
13	10131	Ac	Val	Val	Apns	Dmt	Val	Met	NH_2	77	95
14	10132	Ac	Ala	Val	Apns	Dmt	Val	Met	NH_2	56	96
15	10129	Ac	Leu	Val	Apns	Dmt	Val	Met	NH_2	83	99
16	10130	Ac	Ile	Val	Apns	Dmt	Val	Met	NH_2	87	98
17	10128	Ac	Phe	Val	Apns	Dmt	Val	Met	NH_2	74	95
18	10138	Ac	Gln	Ile	Apns	Dmt	Val	Met	NH_2	85	95
19	10139	Ac	Gln	Leu	Apns	Dmt	Val	Met	NH_2	54	83
20	10140	Ac	Gln	Mta	Apns	Dmt	Val	Met	NH_2	51	96
21	10146	Ac	Gln	Val	Apns	Dmt	Ile	Met	NH_2	74	95
22	10147	Ac	Gln	Val	Apns	Dmt	Leu	Met	NH_2	30	84
23	10148	Ac	Gln	Val	Apns	Dmt	Mta	Met	NH_2	<30	92
24	10149	Ac	Gln	Val	Apns	Dmt	Thr	Met	NH_2	<30	78
25	10156	Ac	Ile	Val	Apns	Dmt	Val	Gln	NH_2	86	95
26	10150	Ac	Ile	Val	Apns	Dmt	Val	Ile	NH_2	78	97
27	10157	Ac	Ile	Val	Apns	Dmt	Val	Val	NH_2	70	96
28	10151	Ac	Ile	Val	Apns	Dmt	Val	Leu	NH_2	54	95
29	10153	Ac	Ile	Val	Apns	Dmt	Val	Phe	NH_2	84	98
30	10166	Ac	Ile	Ile	Apns	Dmt	Ile	Met	NH_2	94	98

[a] HTLV-I PR inhibition (%) at 100 μM of the test compound.
[b] HIV-1 PR inhibition (%) at 50 nM of the test compound.

RT-RH/IN substrates, namely Val, Ala, and Leu (Table 1). We noted a trend in compounds **13**–**15** that inhibitory activity seems to increase with lipophilicity and consequently synthesized compounds **16** and **17**, each, respectively, possessing P_3 Ile or Phe, that would only bring us to conclude that the S_3 pocket is quite accommodating for different residues. Our enzymatic assay results are supported by the evidence that peptidic substrates with a hydrophobic P_3 residue, such as Val, Leu or Phe, exhibited low K_m values.[7] Somewhat satisfied with our outcome, we pursued modifications at the P_2 position (**18**–**20**) in which compound **18** was designed on the P_2 residue of substrates from the Gag/PR and PR/Pol cleavage sites, namely Ile. Compound **19** was synthesized because Leu has similar physicochemical properties as Ile, and observed that a slight alteration at the P_2 position would greatly influence enzymatic activity. Indeed, compound **20**, possessing a less bulky P_2 L-methylthioalanine (Mta), exhibited lower HTLV-I PR activity. It has been reported that, for the case of peptidic substrates, the P_2 position is much less tolerant to substitution than the P_3 or P_4 position, and hydrophobic residues are preferred.[7] Using similar design principles, we explored the P'_2 position with Ile and Leu in compounds **21** and **22**, respectively, to correspond with the PR/Pol and Pro/RT substrates. The activity results for compounds **21**–**24** possessing either a P'_2 Ile, Leu, Mta or Thr follow a similar trend as that observed for compounds **18**–**20** possessing either a P_2 Ile, Leu or Mta, and strongly suggest that the P'_2 position is also a strong determinant for activity at which hydrophobic side chains are preferred. By the time that we arrived at the P'_3 position, we realized that the P_3 Gln residue could be detrimental for our ultimate goal of a cell-penetrable potent HTLV-I PR inhibitor, and adopted our most potent inhibitor at the time, compound **16** possessing a P_3 Ile, as reference. Compounds **25** and **26** possessing either a P'_3 Gln or Ile residue, respectively, to imitate the substrates recognized at the CA/NC, Gag/PR, and PR/Pol cleavage site, accordingly, did not deviate much in inhibitory activity relative to reference compound **16**. To confirm that the P'_3 position is a low determinant for inhibitory activity, the potencies for compounds **27**–**29** possessing either P'_3 Val, Leu or Phe were evaluated. As expected, there were fewer differences in activity.

General observations from substrate specific studies on retroviral aspartic PRs are similar to the trends observed in our inhibitors: the P_4/P'_4 and S_4/S'_4 interactions, which are at the edges of the active site according to Li et al.'s HTLV-I PR X-ray crystallography data,[9] are lower than the P_3/P'_3–S_3/S'_3 interactions; and that the P_3/P'_3 positions are most tolerant to a variety of different side chains when compared with the P_2/P'_2 positions, with the P_1/P'_1 positions being most sensitive to changes.[7] This similarity suggests that our peptidic inhibitors would associate with HTLV-I PR in a similar manner as peptidic substrates.

Compound **30** was designed to take advantage of each optimized residue position, and, as expected, exhibited fairly potent inhibitory activity against HTLV-I PR (94%), surpassing the activities of reference compounds **12** (66%) and **16** (87%). Moreover, this residue optimization study supports our original conclusion from our current peptide chain-length study that a six-residue inhibitor is sufficiently large to bear potent HTLV-I PR inhibitory activity.

Considering that both the HTLV-I and human immunodeficiency virus type 1 (HIV-1) belong to the *Retroviridae* (retrovirus) family, these viruses could be referred to as distant cousins, in which the HTLV-I and HIV-1 PRs share a 28% sequence identity and that the PRs' cleavage regions share a 45% sequence identity.[10] However, several studies have reported that both substrate specificity and inhibition profile of the two enzymes are fairly different.[2,5,11] Moreover, in vitro studies of mainstream HIV-1 PR inhibitors including Indinavir, Nelfinavir, Ritonavir, and Saquinavir failed to show effectiveness in HTLV-I PR.[12] Interestingly, a recent study proved the contrary in that Ritonavir was shown to be effective against ATL both in in vitro and in vivo mouse models.[13] In our current study, we did not find statistically reliable, mathematical relationships between HTLV-I and HIV-1 PR inhibitory activity, in which the coefficient of determination (r^2) was greater than 0.80 (data not shown). However, we did observe that in general, hexapeptidic HTLV-I PR inhibitors (**12**–**30**) usually also exhibit potent HIV-1 PR inhibitory activity. However, hexapeptides exhibiting potent HIV-1 PR inhibition do not necessarily exhibit potent HTLV-I PR inhibitory activities (**22**–**24**). Our observation is supported by a report that HTLV-I PR displays a high degree of specificity over that of HIV-1 PR.[10]

In summary, we used previously reported potent HTLV-I PR inhibitor KNI-10161 (**7**) as a lead compound and performed truncation studies to derive small hexapeptide KNI-10127 (**12**) with some loss in activity, from 94% to 66% inhibition at 100 μM test compound. After performing substitution studies at each amino acid residue position in compound KNI-10127 (**12**), we recovered HTLV-I PR inhibitory potency in inhibitor KNI-10166 (**30**). Rational computer-assisted docking experiments generated a model of KNI-10166 (**30**) in an HTLV-I PR active site that revealed multiple possible hydrogen bond interactions throughout the inhibitor, thereby re-enforcing its high inhibitory potency in HTLV-I PR assay (Fig. 1). We also observed that hexapeptidic inhibitors of HTLV-I PR (**12**–**30**) in general possess potent HIV-1 PR inhibitory activity, but not necessarily the converse.

The synthesis of reference compounds **7** and **11** was previously reported.[6] Compounds **1**–**6**, **8**–**10**, and **12**–**30** were synthesized by Fmoc-based solid phase peptide synthesis. Fmoc-Apns-OH, Fmoc-Dmt-OH, and Fmoc-Mta-OH were synthesized using Fmoc-OSu, while the remaining Fmoc-amino acids were commercially obtained. In each case for compounds **1**–**6**, Fmoc-His(Trt)-OH was loaded onto a 2-chlorotrityl chloride resin in the presence of DIEA. In each case for compounds **8**–**10** and **12**–**30**, the respective C-terminal Fmoc-amino acid was loaded onto a Rink amide AM resin by DIPCDI-HOBt method. In each ensuing Fmoc-deprotection step, 20% piperidine in DMF was employed. In each chain-elongation step, coupling with an appropriate Fmoc-protected amino acid was performed using the DIPCDI-HOBt method. N-Acetylation in compounds **2**, **4**, **6**, and **12**–**30** was performed with acetic anhydride and Et_3N in DMF. Removal of side-chain protecting groups and cleavage from the resin were performed with TFA, *m*-cresol, thioanisole, and water. After preparative HPLC purification, all target compounds (**1**–**30**) were >98% pure by analytical HPLC. The identities of the compounds were confirmed by ESI-Q MS and/or TOF MS. Synthetic yields ranged from 35% to 84%. Recombinant HTLV-I and HIV-1 PR assays were performed according to previously reported procedures.[6] The mathematical correlation between HTLV-I and HIV-1 PR inhibitory activity was calculated with Microsoft Office Excel 2003. Computer-assisted modeling experiments were performed by which the P_1–P'_1 residues of potent HIV-1 PR inhibitor KNI-577 found in PDB 1MRX were merged into the HTLV-I PR inhibitor found in chains A–B of PDB

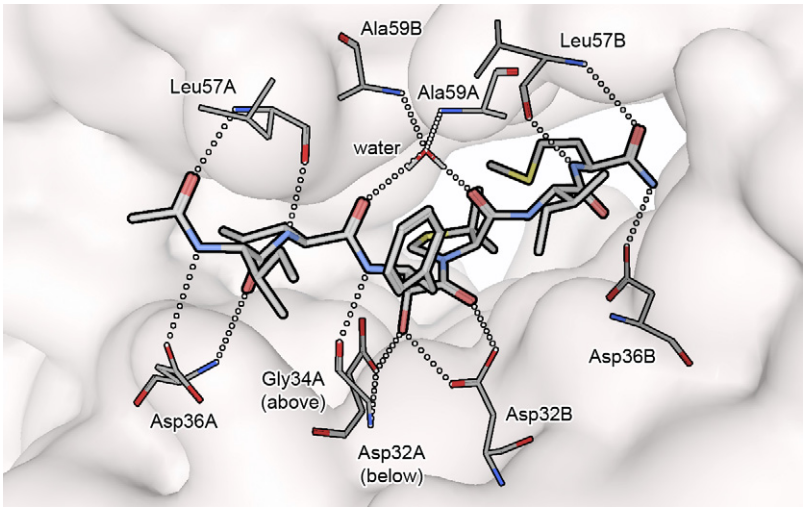

Figure 1. Computer model of KNI-10166 (**30**) in the active site of HTLV-I PR. Circles represent possible hydrogen bond interactions throughout the backbone of KNI-10166 (**30**) and HTLV-I PR's Asp32A, Gly34A, Asp36A, Leu57A, Ala59A, Asp32B, Asp36B, Leu57B, and Ala59B from the dimer. It is noteworthy that a water molecule could mediate the interactions between Ala59A and Ala59B in the hairpin regions of HTLV-I PR's flaps and the inhibitor, and that KNI-10166 (**30**)'s transition-state mimic HMC moiety could interact with catalytic Asp32A and Asp32B.

2B7F, and the model was modified accordingly to resemble KNI-10166 (**30**). The generated model was 'water-soaked' and energy-minimized in an MMFF94x force-field using the 2006.08 release of Chemical Computing Group's Molecular Operating Environment software.

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