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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.
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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

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