KYNOSTATIN (KNI)-227 AND -272, HIGHLY POTENT ANTI-HIV AGENTS: CONFORMATIONALLY CONSTRAINED TRIPEPTIDE INHIBITORS OF HIV PROTEASE CONTAINING ALLOPHENYLNORSTATINE[1,2]

Tsutomu MIMOTO,[a,b] Junya IMAI,[a] Sumitsugu KISANUKI,[a,b] Hiroshi ENOMOTO,[a] Naoko HATTORI,[b] Kenichi AKAJI,[a] and Yoshiaki KISO*,[a]

Department of Medicinal Chemistry, Kyoto Pharmaceutical University,[a] Yamashina-ku, Kyoto 607, Japan and Bioscience Research Laboratories, Nippon Mining Co.,[b] Niizo-minami, Todashi, Saitama 335, Japan

Selective and potent HIV protease inhibitors containing allophenylnorstatine [Apns; (2*S*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] as a transition-state mimic were designed and synthesized. Among them, conformationally constrained tripeptide derivatives, kynostatin (KNI)-227 and -272 (Fig.1), exhibited highly potent antiviral activities against a wide spectrum of HIV isolates. Ready availability due to the simple synthetic procedure and the excellent antiviral properties indicate that KNI-227 and KNI-272 are promising candidates as selective anti-AIDS drugs.

KEYWORDS HIV protease inhibitor; anti-HIV agent; kynostatin; peptide synthesis; hydroxymethylcarbonyl isostere; transition-state mimic; allophenylnorstatine; AIDS

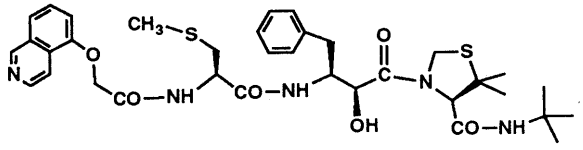
The human immunodeficiency virus (HIV) codes for an aspartic protease known to be essential for retroviral maturation and replication. The HIV protease can recognize Phe-Pro and Tyr-Pro sequences as the virus-specific cleavage site. These features provided a basis for the rational design of selective HIV protease-targeted drugs for treatment of AIDS.[4]

We have already reported the novel class of HIV protease inhibitors[3,5,6] containing allophenylnorstatine [Apns; (2*S*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid] with a hydroxymethylcarbonyl (HMC) isostere[7] designed from the substrate transition state (Fig.2). The critical hydroxyl group as a transition-state mimic interacts with the aspartic acid carboxyl groups of the HIV protease active site, and the stereochemistry of the hydroxyl group was significant for the inhibition.[6]

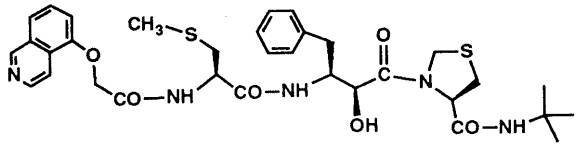
Having identified the tripeptide derivative KNI-102[3,6] (**1**) (Table I) as a lead compound, we undertook a study of lead optimization to find a highly selective and potent HIV protease inhibitor, KNI-174 (**20**), with anti-HIV activity.[3] Further structure-activity relationship study considering the penetration across cell membrane and the behaviour *in vivo* resulted in the generation of highly potent protease active site-targeted anti-HIV agents. In this paper, we describe Apns-containing HIV protease inhibitors, kynostatin (KNI)-227 (**25**) and kynostatin (KNI)-272 (**19**) (Fig.1), which exhibit extremely potent antiviral activity against a wide spectrum of HIV isolates.

For the lead optimization of KNI-102, we determined the structural requirements for potent activity at each subsite (Table I). At the P_2' residue, the t-butyl amide was more suitable than the small (compound **3**) or bulky (compound **4**) group. The amide bond was 10-fold preferable to the ester bond (compound **2**), in contrast to the case of hydroxyethylamine (HEA)-type inhibitors.[8]

The most interesting results were obtained at the P_1' residue. The pyrrolidine ring of proline was more suitable than the expanded piperidine ring of pipecolinic acid (compound **5**), in contrast to the case of HEA type inhibitor.[8] Molecular modeling techniques[9] indicated that the hydroxyl group of hydroxymethylcarbonyl (HMC) isostere[10] played a very important role for interaction with the protease. The expanded piperidine ring seems to interfere with the interaction of the hydroxyl group of HMC isostere and the protease, which implies a conformational restriction in the HMC isostere-containing



25 (KNI-227)



19 (KNI-272)

Fig.1. Chemical Structures of KNI-227 and KNI-272

peptides, as discussed previously.[6] The replacement of the pyrrolidine by the thiazolidine (KNI-125; **7**), dimethylpyrrolidine (compound **8**), or dimethylthiazolidine (KNI-162; **9**) enhanced the protease inhibitory activity. The constrained conformation of these HMC-containing peptides might be responsible for the high activity.

The naphthyl group (KNI-144; **10**) of P_3 residue fitted favorably in the large hydrophobic S_3 subsite, and the aryloxyacetyl type groups were preferred (compounds **10** *vs.* **11**). At the P_2 position, Asn residue could be replaced by L-methanesulfonylalanine (Msa) residue [KNI-151 (**16**) and KNI-170 (**21**)] or L-methylthioalanine (Mta) residue [KNI-217 (**17**) and KNI-225 (**22**)], with a slight improvement in activity, in contrast to the case of HEA type inhibitor.[8]

Combinations of each preferred side chain led to highly selective and potent HIV protease inhibitors, such as KNI-174 (**20**; IC_{50} = 2.8nM), KNI-170 (**21**; IC_{50} = 2.6nM) and KNI-225 (**22**; IC_{50} = 2.7nM), with little inhibition of other aspartic proteases such as porcine pepsin (IC_{50} > 10,000nM for each inhibitor) and human plasma renin (IC_{50} > 100,000nM for each inhibitor). Preliminary data have shown that these compounds exhibit potent antiviral activities against HIV-1 in $CD4^+$ ATH8 cells.[12]

The behaviors of compounds *in vivo*, such as penetration across the cell membrane and non-specific adsorption in blood, are important factors for the *in vivo* antiviral activity. Therefore, considering the subtle balance of lipophilicity-hydrophilicity and molecular size, we incorporated the 5-isoquinolinyloxyacetyl (iQoa) moiety at the P_3 position and combined it with each preferred side chain. Such modifications resulted in the highly active compounds [KNI-227 (**25**) and KNI-272 (**19**) (IC_{50}= about 0.01μM for both compounds)] against clinical HIV-1 isolates in phytohemagglutinin-stimulated peripheral blood mononuclear cells *in vitro*,[12] which may relate to the possible *in vivo* antiviral activity. These antiviral activities against clinical HIV-1 isolates appear to be more than 10-fold potent compared to a C_2 symmetric protease inhibitor, A-77003[13] on the basis of molarity, although more detailed comparative studies are neccesary.

Table I. HIV-1 Protease Inhibition of Tripeptides[a]

Number		P_3	P_2	P_1	P_1'	P_2'	IC_{50}(nM)[b]
1	(KNI-102)	Z-	Asn-	Apns-	Pro-	$NHBu^t$	89
2		Z-	Asn-	Apns-	Pro-	OBu^t	868
3		Z-	Asn-	Apns-	Pro-	$NHPr^i$	320
4		Z-	Asn-	Apns-	Pro-	NHCh	572
5		Z-	Asn-	Apns-	Pip-	$NHBu^t$	450
6		Z-	Asn-	Apns-	Tic-	$NHBu^t$	>1,000
7	(KNI-125)	Z-	Asn-	Apns-	Thz-	$NHBu^t$	31
8		Z-	Asn-	Apns-	Dmp-	$NHBu^t$	24
9	(KNI-162)	Z-	Asn-	Apns-	Dmt-	$NHBu^t$	3.5
10	(KNI-144)	Noa-	Asn-	Apns-	Pro-	$NHBu^t$	12
11		Nmoc-	Asn-	Apns-	Pro-	$NHBu^t$	24
12		Fmoc-	Asn-	Apns-	Pro-	$NHBu^t$	45
13		Dcoa-	Asn-	Apns-	Pro-	$NHBu^t$	28
14		Qc-	Asn-	Apns-	Pro-	$NHBu^t$	20
15	(KNI-154)	Noa-	Asn-	Apns-	Thz-	$NHBu^t$	8.8
16	(KNI-151)	Noa-	Msa-	Apns-	Thz-	$NHBu^t$	4.0
17	(KNI-217)	Noa-	Mta-	Apns-	Thz-	$NHBu^t$	3.2
18	(KNI-273)	iQoa-	Msa-	Apns-	Thz-	$NHBu^t$	7.2
19	**(KNI-272)**	iQoa-	Mta-	Apns-	Thz-	$NHBu^t$	6.5
20	(KNI-174)	Noa-	Asn-	Apns-	Dmt-	$NHBu^t$	2.8
21	(KNI-170)	Noa-	Msa-	Apns-	Dmt-	$NHBu^t$	2.6
22	(KNI-225)	Noa-	Mta-	Apns-	Dmt-	$NHBu^t$	2.7
23	(KNI-208)	*m*Bpoa-	Asn-	Apns-	Dmt-	$NHBu^t$	2.2
24	(KNI-226)	*m*Bpoa-	Mta-	Apns-	Dmt-	$NHBu^t$	2.3
25	**(KNI-227)**	iQoa-	Mta-	Apns-	Dmt-	$NHBu^t$	2.3

a) These tripeptide derivatives were synthesized by essentially the same procedure as described previously[3,6] (for example, see Chart 1). b) Protease inhibitory activity was determined using a synthetic [$Ala^{67,95}$]-HIV-1 protease,[5] as previously reported.[5,6]
Abbreviations: Z = benzyloxycarbonyl, Apns = (2*S*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid, Bu^t = t-butyl, Pr^i = isopropyl, Ch = cyclohexyl, Pip = L-pipecolinic acid, Tic = L-tetrahydroisoquinolinecarboxylic acid, Thz = L-thiazolidine-4-carboxylic acid, Dmp = L-3,3-dimethylpyroridine-2-carboxylic acid, Dmt = L-5,5-dimethylthiazolidine-4-carboxylic acid, Noa = 1-naphthoxyacetyl, Nmoc = 1-naphthylmethyloxycarbonyl, Fmoc = 9-fluorenylmethoxycarbonyl, Dcoa = 4,4'-dichlorobenzhydryloxyacetyl, Qc = quinolin-2-ylcarbonyl, Msa = L-methanesulfonylalanine, Mta = L-methylthioalanine, iQoa = 5-isoquinolinyloxyacetyl, *m*Bpoa = *m*-biphenyloxyacetyl.

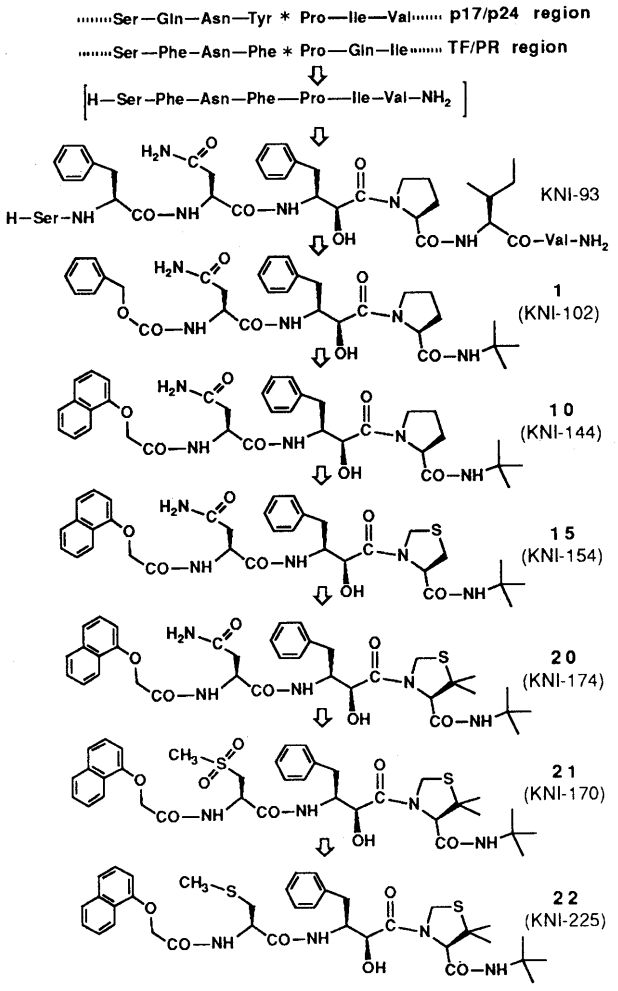


Fig.2. Design of Selective and Potent HIV Protease Inhibitors

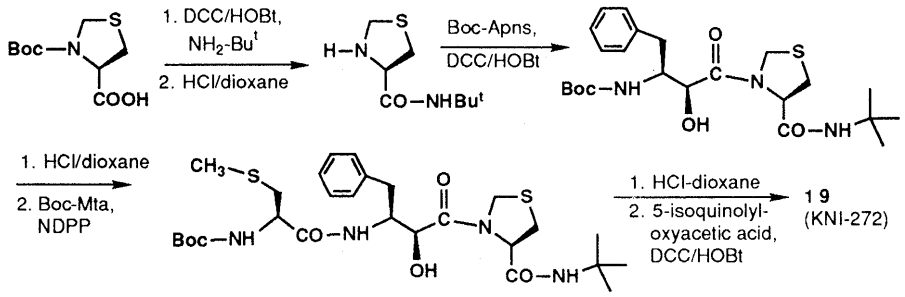


Chart 1. Synthetic Scheme of KNI-272 (**19**)
Pure compound **19** was conveniently synthesized by the solution method in a stepwise manner and readily obtained. Abbreviations: Boc=t-butoxycarbonyl, DCC=dicyclohexylcarbodiimide, HOBt=N-hydroxybenzotriazole, NDPP=norborn-5-ene-2,3-dicarboximido diphenyl phosphate.[14]

Two compounds, KNI-227 (**25**) and KNI-272 (**19**), were highly potent HIV-1 protease inhibitors (Table I) with little inhibition of other aspartic proteases such as human plasma renin (IC_{50}>100μM) and porcine pepsin(IC_{50}>10μM), and preliminary data have shown the potent antiviral activities against the infectivity and cytopathic effect of HIV strains, including HIV-1_{LAI}, HIV-1_{RF}, HIV-1_{MN} and HIV-2_{ROD}, as tested in $CD4^+$ ATH8 cells.[12] From the viewpoint of the action mechanism, the active site-targeted HIV protease inhibitors have reason to exhibit activities against a wide spectrum of HIV strains, including HIV-2.

Interestingly, a relatively low-lipophilic and small-sized tripeptide derivative, KNI-272 (**19**), combined with iQoa moiety and L-thiazolidine-4-carboxylic acid (Thz) residue, exhibited highly potent antiviral activities and low cytotoxicity (TC_{50} > 80μM). Ready availability due to the simple synthetic procedure of the tripeptide derivatives and the excellent antiviral properties indicate that KNI-227 and KNI-272 are promising candidates as selective anti-AIDS drugs.

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10) Recently, it has been confirmed by several researchers[11] that the HMC isostere is an effective transition-state mimic in HIV protease inhibitors.

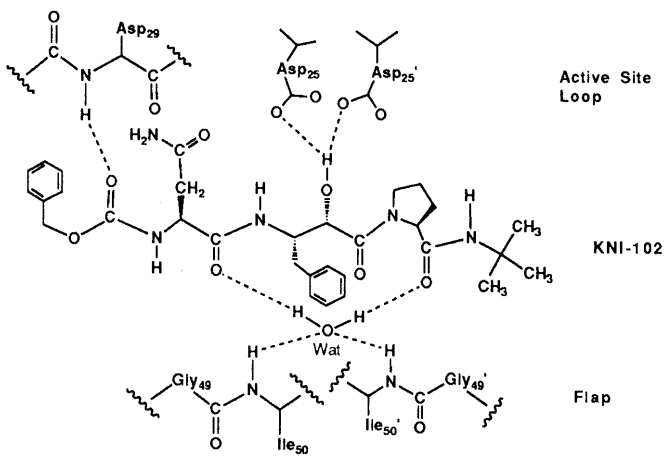

Fig.3. KNI-102 in the Active Site of HIV Protease

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