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Additional interaction of allophenylnorstatine-containing tripeptidomimetics with malarial aspartic protease plasmepsin II

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Abstract—Based on a highly potent allophenylnorstatine-containing inhibitor, KNI-10006, against the plasmepsins of *Plasmodium falciparum*, we synthesized a series of tripeptide-type compounds with various N-terminal moieties and evaluated their inhibitory activities against plasmepsin II. Certain phenylacetyl derivatives exhibited extremely high affinity with K_i values of less than 0.1 nM suggesting successful hydrophobic interactions.

The most lethal malaria parasite, *Plasmodium falciparum*, increasingly develops resistance to available drugs such as chloroquine or sulfadoxine-pyrimethamine.[1] Drug resistance is one of the reasons for mortality due to malaria, reaching up to 3 million people every year. Malaria parasites invade the human host, who had been bitten by the *Anopheles* mosquito carrier, through hematocyte multiplication and proliferate during the erythrocyte cycle. In the cycle, hemoglobin is transported to acidic food vacuoles and then cleaved by proteases into small peptides to obtain amino acids as nutrients. This hemoglobin degradation is essential for the parasites' propagation.[2] In the case of *P. falciparum*, four aspartic proteases, named plasmepsins (Plms), participate in the digestion.[3] Recent studies suggested the importance of inhibiting all four Plms to suppress parasite multiplication.[4] Plm II has become a promising focus for new anti-malarial drug design since the time its crystallographic data first became available.[5] Thus far, several groups have reported Plm inhibitors with potent inhibitory activities.[6] Our design of Plm inhibitors is based on the hydroxymethylcarbonyl (HMC) isostere as an ideal transition-state mimic established in renin and HIV protease inhibitor studies.[7] We have reported small peptidic compounds incorporating an allophenylnorstatine [Apns; (2*S*,3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid]-containing scaffold, that exhibited potent Plm II inhibitory and anti-malarial activity.[8] Among these analogues, dipeptidomimetic KNI-10006 exhibited highly potent inhibitory activity against Plm II (Fig. 1). KNI-10006 also exhibited high potency against Plm I, IV, and histo-aspartic protease.[9]

Structure–activity relationship (SAR) studies at the position of KNI-10006 revealed a significant P_2' contribution of the (1*S*,2*R*)-aminoindanol moiety to the tight binding.[10] The inhibitory activity of KNI-10006, however, was attenuated in *P. falciparum*-infected erythrocyte cultures. Our attention was directed to modifying other positions in KNI-10006. Modifications of the dipeptidic structure failed to obtain compounds with more potent inhibitory activity against Plm II (data not shown). In an attempt to diversify the development of Apns-containing Plm inhibitors, we herein report SAR on the N-terminal moiety of tripeptide-type compounds containing Apns.

KNI-272[7c,d] was the first compound tested as a tripeptidic Plm II inhibitor containing Apns.[8] Previous studies on dipeptidic Plm II inhibitors stimulated us to examine KNI-227,[7c] possessing dimethyl groups in the P_1' residue, specifically, Dmt [(*R*)-5,5-dimethyl-1,3-thiazoli-

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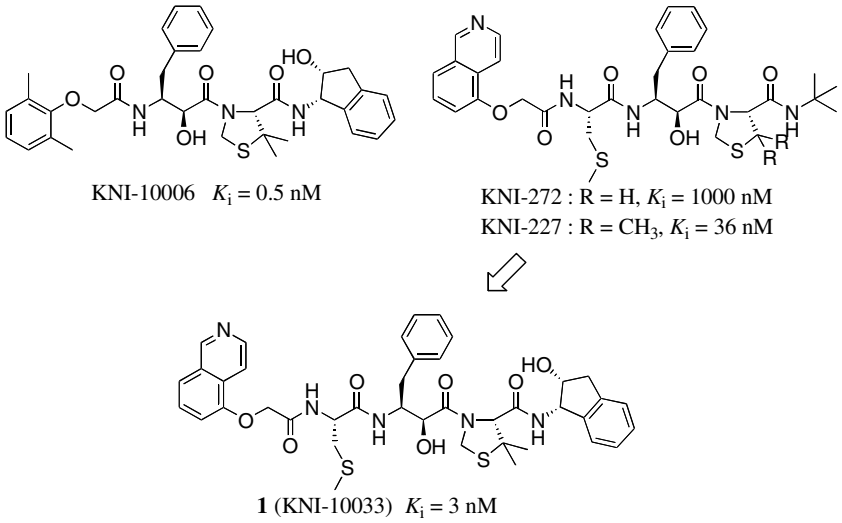
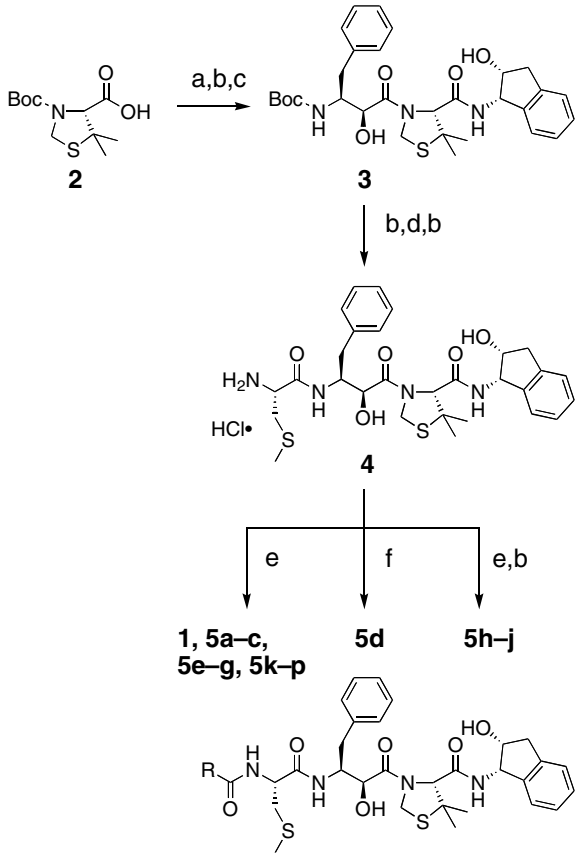


Figure 1. Structures and Plm II inhibition constants of KNI-compounds.

dine-4-carboxylic acid] (Fig. 1). KNI-227 exhibited Plm II inhibitory activity more potently than KNI-272 and had improved Plm II selectivity (~50-fold) over human cathepsin D. Replacements of the P_2 (*R*)-methylthioalanine (Mta) with hydrophobic or hydrophilic residues were not effective in maintaining inhibitory activity (data not shown). Introduction of (1*S*,2*R*)-1-amino-2-indanol at the P_2' position of KNI-227 gave compound **1** (KNI-10033) exhibiting potent inhibitory activity with a K_i value of 3 nM (Fig. 1). Although the activity in *P. falciparum*-infected erythrocyte cultures was slightly more desirable (EC_{50} = 3.1 μM) than that of KNI-10006 (EC_{50} = 6.8 μM), the enzyme inhibitory activity of **1** was less potent than that of KNI-10006. Therefore, our attention was shifted to modify the *N*-acyl group of Mta.

Synthetic routes of the N-terminal modified tripeptidic compounds are shown in Scheme 1. Intermediate **4** was synthesized from Boc-Dmt-OH (**2**) as previously reported[10] with subsequent peptide couplings with benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) or *N*-ethyl-*N*′-[3-(dimethylamino)propyl]carbodiimide (EDC) plus 1-hydroxybenzotriazole (HOBt) method and deprotections of Boc group using HCl-dioxane. In most cases, compounds **5** were synthesized by coupling with corresponding acids using the BOP reagent. In the case of compound **5d**, cyclohexyl succinimidylcarbonate was reacted at rt. For amino-substituted phenylacetyl derivatives (**5h–j**), Boc-amino-substituted phenylacetic acids were used and finally deprotected. The pure products were obtained after purification by preparative HPLC and identified by MALDI-TOF MS. Enzyme inhibitory activities against Plm II and HIV-1 protease were evaluated as previously described.[8,10]

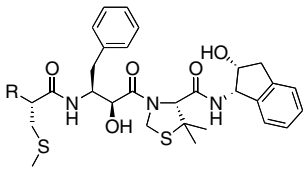
We introduced N-terminal moieties that were known to be effective in HIV-1 protease inhibition in previous studies.[7e] In spite of their potent HIV-1 PR inhibitions, similar to **1**, compounds **5a** and **5b** exhibited less potent

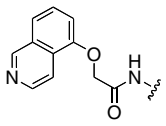
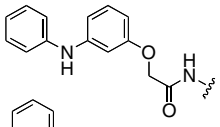
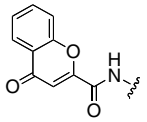
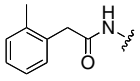
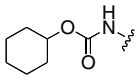


Scheme 1. Reagents: (a) (1*S*,2*R*)-1-amino-2-indanol, BOP, Et_3N DMF; (b) 4 N HCl/dioxane, anisole; (c) Boc-Apns-OH, EDC·HCl, HOBt, Et_3N, DMF; (d) Boc-Mta-OH, EDC·HCl, HOBt, Et_3N, DMF; (e) RCOOH, BOP, Et_3N, DMF; (f) cyclohexyl succinimidylcarbonate, Et_3N, DMF. Mta = (*R*)-methylthioalanine.

inhibitory activity against Plm II (Table 1). We assumed that an intramolecular hydrogen bond between the oxygen of the ether moiety and NH group of Mta was formed. This conformational constraint would be preferred for binding with HIV-1 protease but would interrupt the bindings within the pockets of Plm II.

Table 1. Inhibitory activity of N-terminal modified compounds



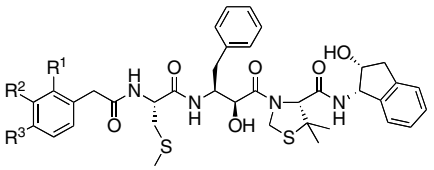
Compound	R	Plm II K_i(nM)	HIV-1 PR % inhibition at 50 nM
1 (KNI-10033)		3	99
5a (KNI-10061)		15	98
5b (KNI-10062)		9	99
5c (KNI-10232)		0.2	83
5d (KNI-10233)		5	56

Therefore, we introduced two other moieties in compounds **5c** and **5d** that would not form the intramolecular hydrogen bond interaction present in compounds **1**, **5a**, and **5b**. We identified compound **5c** (KNI-10232) that possessed more potent Plm inhibitory activity (K_i = 0.2 nM) than KNI-10006.

We focused on the phenylacetyl structure of **5c** and changed its *o*-methyl substituent. We introduced methyl, amino, hydroxyl, and methoxy substituents at each position on the phenyl ring (Table 2). These compounds possessed moderate HIV-1 protease inhibition due to a lack of the intramolecular hydrogen bond interaction at the N-terminal moieties. As a result, highly potent activities were shown only in the cases of *o*-methyl (**5c**), *p*-amino (**5i**), and *p*-hydroxyl (**5l**) substitutions. The latter two compounds exhibited extremely potent activities ($K_i \leqslant 0.1$ nM) suggesting possible hydrogen bond interactions at the *p*-position to direct the orientation of the phenyl ring. Plm II favored none of the *m*-substitutions, thereby implying steric hindrance at the *m*-position. Interestingly, the removal of all substitutions maintained highly potent activity (**5p**). Although differences in their affinities are not clear, we believe that successful hydrophobic interactions near the phenyl ring play a key role.

The speculative interaction of **5i** (KNI-10333) with Plm II was confirmed by computational simulation (Fig. 2). The inhibitor was manually docked into Plm II (PDB ID, 1SME) by using a modeling package (MOE

Table 2. Inhibitory activity of phenylacetyl derivatives against Plm II and HIV-1 PR



Compound	R^1	R^2	R^3	Plm II K_i (nM)	HIV-1 PR % inhibition at 50 nM
5c (KNI-10232)	CH_3	H	H	0.2	83
5e (KNI-10332)	H	CH_3	H	18	87
5f (KNI-10313)	H	H	CH_3	5	88
5g (KNI-10372)	NH_2	H	H	4	86
5h (KNI-10368)	H	NH_2	H	12	76
5i (KNI-10333)	H	H	NH_2	$\leqslant$0.1	68
5j (KNI-10341)	OH	H	H	16	86
5k (KNI-10342)	H	OH	H	4	84
5l (KNI-10343)	H	H	OH	$\leqslant$0.1	88
5m (KNI-10314)	OCH_3	H	H	9	81
5n (KNI-10316)	H	OCH_3	H	17	88
5o (KNI-10315)	H	H	OCH_3	4	91
5p (KNI-10395)	H	H	H	$\leqslant$0.1	85

2006.08, Chemical Computing Group Inc., Montreal, Canada). Several energy minimization processes with an MMFF94x force field were additionally performed including a water soak around the inhibitor. In the minimization steps, hydrogen bond interactions of HMC with the two catalytic Asps were kept similar to that observed in HIV protease complexes (PDB ID, 1HPX, and 1KZK), that is, the hydroxyl group of HMC interacted with the carboxylate anion of one of the Asp, and the carbonyl interacted with the carboxylic acid proton of the other Asp.[11] An energetically favored conformation of **5i** was obtained in Plm II, with Asp34 being protonated (Fig. 2A). Most of the hydrogen bond interactions were kept similar to a previous report[10] with differences near the phenylacetyl-Mta moiety. The N-terminal phenyl moiety was surrounded by Phe 11, Gln12, Ile13, and Met15, and the CH_2 moiety had hydrophobic contact with the side-chain of Met15 and was a little distant from the side chain of Thr 114. We observed hydrogen bond interactions of the *p*-amino group with two Gln residues. In a recent report by Åqvist's group,[12] other possible conformations in the opposite direction were suggested using X-ray crystallographic data (PDB, 2ANL) of an Apns-containing compound KNI-764 and Plm IV from *Plasmodium malariae* (PmPM IV).[13] Sequence identity between Plm II and PmPM IV is 72% including differences around binding pockets such as Val78/Gly and Tyr192/Phe. One of the possible conformations of **5i** in Plm II with a protonated Asp214 is illustrated in Figure 2B. Dramatic movement of Val78 in the flap region of Plm II accommodated for the benzyl group of Apns and N-terminal group, and disrupted closing of the flap. On the other hand, some hydrogen bond interactions were consistent with those suggested by Åqvist et al. The latter aminoindanol moiety could form hydrogen bond interactions with Ser218, and not with Tyr192 in Figure 2A. The interaction energy, which

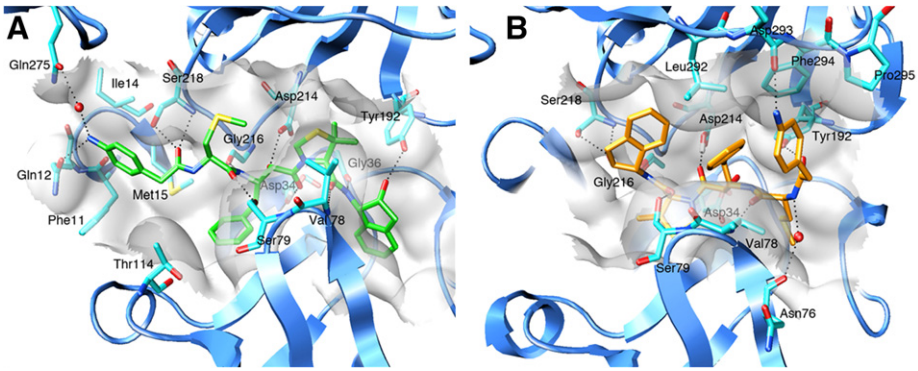


Figure 2. Two speculative binding models of compound **5i** [green in (A) or orange in (B)] and Plm II (blue ribbon). One of the catalytic two aspartates was intentionally protonated [Asp34 in (A), Asp214 in (B)].

is the sum of the electrostatic and van der Waals energies calculated by MOE,[14] between inhibitor and enzyme of the latter conformation (Figure 2B, −152 kcal/mol) was less favorable than that of the former (Figure 2A, −184 kcal/mol) because the N-terminal group of the latter was surrounded mostly by water molecules, and partially by flap and pocket residues (Val78, Phe294, and Pro295).

We evaluated the inhibitory activity of compound **5c** against *P. falciparum*. The EC_{50} value in erythrocyte cultures was 4.7 μM. This significant difference from the K_i value (0.2 nM) suggests a need to modify its chemical properties such as decreasing the number of amide bonds, or improving hydrophobic–hydrophilic balance for membrane permeability to deliver inhibitor to the target Plms.

In conclusion, we synthesized a series of Apns-containing Plm inhibitors at tripeptidic size. Starting from KNI-272, replacements with P_1' dimethyl groups, P_2' aminoindanol and modifications of the N-terminal group led to compounds exhibiting more potent activity than KNI-10006, that are more than 10 thousand times more potent than KNI-272. Evaluations of their anti-malarial activity and further modifications using hydrophobic N-terminal moieties to develop anti-malarial agents are under investigation.

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