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Novel 3-phenylpropane-1,2-diamine derivates as inhibitors of aminopeptidase N (APN)

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ABSTRACT

Aminopeptidase N (APN) is an essential peptidase involved in the process of tumor invasion and metastasis. Here we describe a novel class of inhibitor with 3-phenylpropane-1,2-diamine as scaffold to APN. Preliminary activity evaluation with enzyme inhibition studies showed that compound **12i** exhibited potent and selective inhibitory activity towards APN with the IC_{50} value 15.5 ± 1.2 μM.

1. Introduction

Aminopeptidase N (APN) is a Zn^{2+}-dependent exopeptidase that is expressed by monocytes, myeloid, epithelial cells of the intestine and kidney, fibroblasts, endothelial cells and tumor cells.[1] Being responsible for the cleavage the N-amino group from polypeptide chains, APN plays a key role in physiological processing and degradation of peptides and proteins, and alterations in their activity have been associated with various pathological disorders.[2–5]

Several excellent reviews on natural and small molecule inhibitors of APN have been published.[6–8] Of these inhibitors, the best well known is Bestatin, which was isolated by Umezawa et al. in a search for low molecular weight inhibitors for hydrolytic enzymes on the surfaces of cells.[9]

In recent years, the 3D-structures of APN have been investigated by the X-ray crystallographic studies on the co-crystal of the enzyme and various inhibitors.[10–12] Our group had reported some new APN inhibitors, such as 3-galloylamido-*N*′-substituted 2,6-piperidinedione-*N*-acetamide peptidomimetics,[13] L-lysine derivates,[14] L-iso-glutamine derivatives,[15] and AHPA (β-amino-α-hydroxyl-phenylbutanoic acid) derivates.[16] In the previous study, we reported that one AHPA derivative, AHPA-Val, exhibited better inhibitory activity against APN than that of Bestatin. The docking studies showed that the amino group and carbonyl group of AHPA-Val were chelated to the zinc ion of APN and the aryl ring of AHPA-Val inserted deeply into the extended hydrophobic pocket.[16]

Our group has studied the binding site and catalytic domain of APN based on the co-crystal complex of APN and Bestatin reported by Kiyoshi,[11] the binding site of the APN with Bestatin can be divided to three part, part A is a hydrophobic pocket which interact with phenyl group of Bestatin; part B is the zinc binding group (ZBG) and part C is another hydrophobic pocket in other side. Additionally, part C can be divided into pocket C and pocket C′ in details. However, Bestatin did not show well interaction with part C.[6,14,18]

In our ongoing work, we wanted to develop a novel scaffold based on the AHPA structure and can occupy the part C. Additionally, different building block (such as the residue group of amino acid or carboxylic acid) will be introduced to interact with the hydrophobic pocket A of APN. In this article, we described the synthesis and enzymatic evaluation of the 3-phenylpropane-1,2-diamine derivates and docking studies of the interaction are also discussed.

2. Chemistry

The target compounds were synthesized efficiently following the procedures as shown in Scheme 1. The starting material compound **1** (2*R*)-2-[(*tert*-butoxycarbonyl)amino]-3-phenyl-propano was prepared from D-phenylalanine according to the literature.[17]

The hydroxyl group of compound **1** was converted to mesyl group by methanesulfonyl chloride and then reacted with sodium

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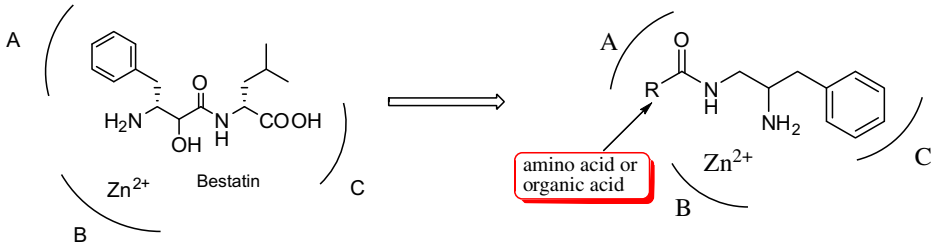


azide to generate azide **3**. The key intermediate **4** was obtained by the disoxidation of compound **3**.

The Boc-amino acids **6** were activated with isobutyl chloroformate and N-methylmorpholine and then coupled with compound **4** to yield **7a–7j**. The Boc-protecting group can be easily removed by 3 N HCl in ethyl acetate to give hydrochloride salts **8a–8j. Compounds 12a–12j** were synthesized similar to **8a–8j**.

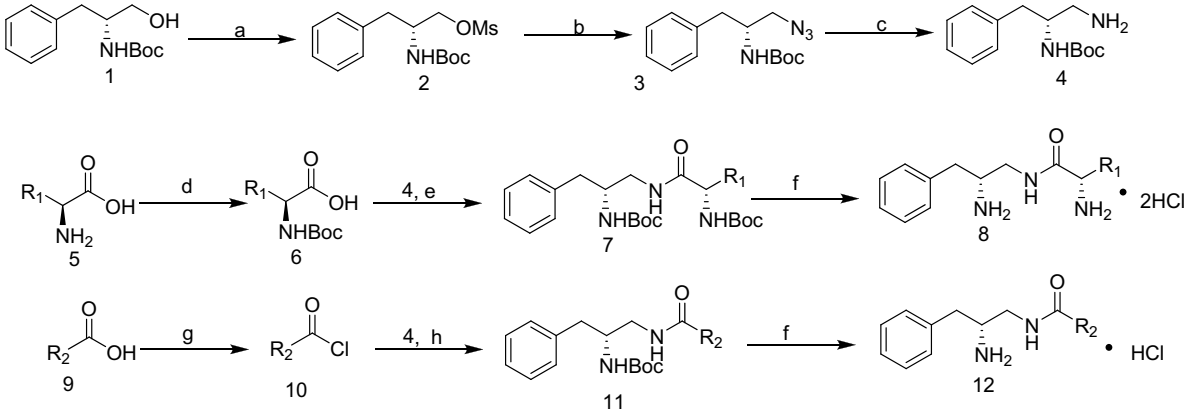
3. Results and discussion

The preliminary pharmacological studies have been investigated on enzymatic inhibition of APN and MMP-2 and the result listed in Table 1. Similar to APN, MMP-2 is also a zinc-dependent metalloproteinase involved in the process of tumor invasion and metastasis. MMP-2 is an endopeptidase which is different with APN, an exopeptidase. To observe the selectivity of these target compounds against two enzyme family, the target compounds were assayed for the inhibitory activities on APN and MMP-2.

As shown in the enzyme assay results, most compounds exhibited a better inhibitory activity on MMP-2 than that of APN. This phenomenon was also shown in our previous studies 3-galloylamido-*N*′-substituted-2,6-piperidinedione-*N*-acetamide peptidomimetics[13] and L-lysine derivates.[14] This result possibly resulted from the differences between the structure of active site of APN and MMP-2. According to the binding site of APN (PDB code: 1HS6) and MMP-2 (PDB code: 1HOV, 1CK7, and 1QIB), Wang et al.[14] showed that the active site of APN was more deeper than the active site of MMP-2, which may lead to the difficulty to access the active site of APN compared with the binding with MMP-2 in most compounds.

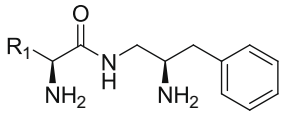
According to the result of enzyme inhibition, compounds **8f**, **8g**, **8j**, and **12i** exhibited most active toward APN among these target compounds. The IC_{50} values for these APN inhibitors range between 15 and 50 μM. Interestingly, these four compounds also showed more potent toward APN than that against MMP-2. The structure in R_1 or R_2 position contains the essential functional group which could interact with the zinc ion in the APN catalytic site, such as amino, hydroxyl and thiol. However, all of these compounds showed a little less activity than that of Bestatin. This phenomenon may result from the difference of the ZBG and the active conformation between the Bestatin and the target compounds we designed. Additionally, the effects of **8f**, **8g**, **8j**, and **12i** on the HL-60 cell proliferation compared with Bestatin were shown in Fig. 1. Cell viability was assessed by MTT method. Compound **12i** and **8j** exhibited the inhibitory effect of cell proliferation with IC_{50} values of 0.63 ± 0.08 mM and 1.09 ± 0.12 mM, respectively, which showed better potency than that of Bestatin with IC_{50} value of 1.65 ± 0.09 mM. These result suggested that compound **12i** exhibit the most potent and selective inhibitory activity towards APN with the IC_{50} value in micromole range. It is necessary to investigate the structure of tripeptide analogue compound **12i** which interact favorably with APN.

In order to investigate the interaction of our target compound with APN, the most active compound **12i** was built and docked into the active site of APN (PDB code: 2DQM) using Sybyl7.0. The binding studies showed that the carbonyl group and amino group of compound **12i** bind to the zinc ion and form a five-membered ring in the active pocket with distance of 2.04 Å and 1.97 Å, respectively. According to the structure, the C′ pocket of APN contains tyrosine residues. This gives favorable interactions with phenyl group of the backbone because of the increase of hydrophobic contact area and possible interactions between the benzene ring of **12i** and the C′ pocket residues. Additionally, the terminal benzene ring of the substituent inserted to the pocket A and another phenyl group of the substituent R_2 interact with the pocket C (Fig. 2a). Compound **12i** also formed hydrogen bonds with His^{297} (<2.98 Å), Glu^{298} (<3.19 Å) and His^{301} (<2.38 Å) which are the essential amino acids of the conserved sequence ($HEXXHX_{18}E$) in the catalytic domain that is well conserved in peptidase M1 family.[19] In addition, the carbonyl group of compound **12i** could interact with the hydroxyl group of Tyr^{381} by hydrogen bond which would be benefit to stabilize of the reaction intermediate with the zinc ion[11] (Fig. 2b)



Scheme 1. Reagents and conditions: (a) MsCl/THF, 0 °C; (b) NaN_3/DMF; (c) Mg/MeOH; (d) $(Boc)_2O$/DCM, Et_3N, 0 °C; (e) isobutyl chloroformate, *N*-methylmorpholine, THF, −15 °C; (f) HCl/EtOAc; (g) oxalyl chloride/DCM, 0 °C; (h) Et_3N, THF, 0 °C.

Table 1
The structures and IC_{50} values of target compounds



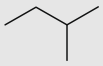
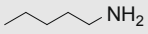
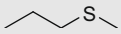
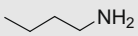
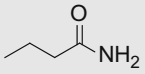
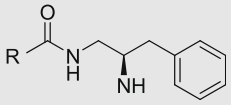
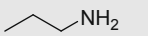
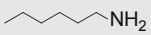
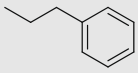
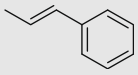
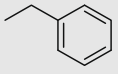
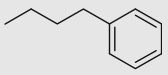
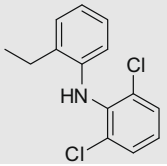
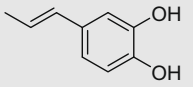
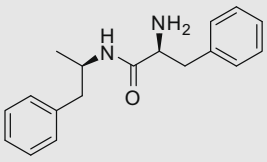
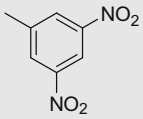
Compound	R_1	IC_{50}[a] (μM)	
		APN	MMP-2
8a	H	>1000	23.5 ± 1.2
8b		142.4 ± 5.3	170.2 ± 2.3
8c		476.0 ± 1.9	151.4 ± 3.6
8d		164.2 ± 2.3	99.7 ± 2.2
8e		118.7 ± 1.5	112.1 ± 3.5
8f		33.7 ± 1.1	201.9 ± 2.9
8g		50.5 ± 0.9	219.4 ± 2.8
8h	CH_3	>1000	771.9 ± 3.9
8i		>1000	802.3 ± 6.5
8j		21.1 ± 0.8	78.8 ± 1.8
			
	R_2		
12a		408.6 ± 4.6	11.2 ± 0.8
12b		164.3 ± 3.2	99.7 ± 2.6
12c		289.5 ± 2.7	546.8 ± 3.4
12d		595.8 ± 2.8	211.3 ± 3.4
12e		>1000	632.4 ± 3.9
12f		>1000	221.0 ± 2.3
12g		>1000	>1000

Table 1 (*continued*)

Compound	R_1	IC_{50}[a] (μM)	
		APN	MMP-2
12h		491.8 ± 2.7	328.5 ± 1.9
12i		15.5 ± 1.2	182.9 ± 2.7
12j		204.5 ± 3.5	100.7 ± 3.4
Bestatin	3.1 ± 0.6	162.0 ± 4.8	

[a] Each value represents the mean of three experiments, standard deviation is given.

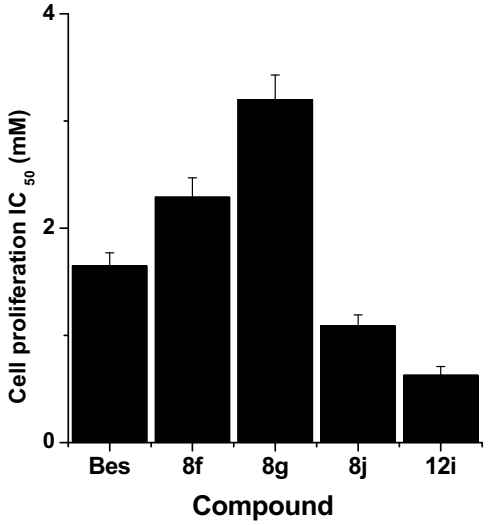


Figure 1. Effects of Bestatin and compounds **8f**, **8g**, **8j**, and **12i** on the HL-60 cell line proliferation. Each column represents the mean with SE of five independent experiments.

4. Conclusions

In summary, we demonstrated a new family of APN inhibitor origin with the scaffold 3-phenylpropane-1,2-diamine. Most of the compounds possess potent activity toward APN and the most potent compound, **12i**, exhibited good enzymatic inhibition and selectivity to APN. This feature may offer one lead compound and could be used to design more potent APN inhibitors in the future.

5. Experimental

5.1. Synthetic methods and spectroscopic details

Unless specified otherwise, all starting material, reagents and solvents were commercially available. All reactions except those in aqueous media were carried out by standard techniques for the exclusion of moisture. All reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visu-

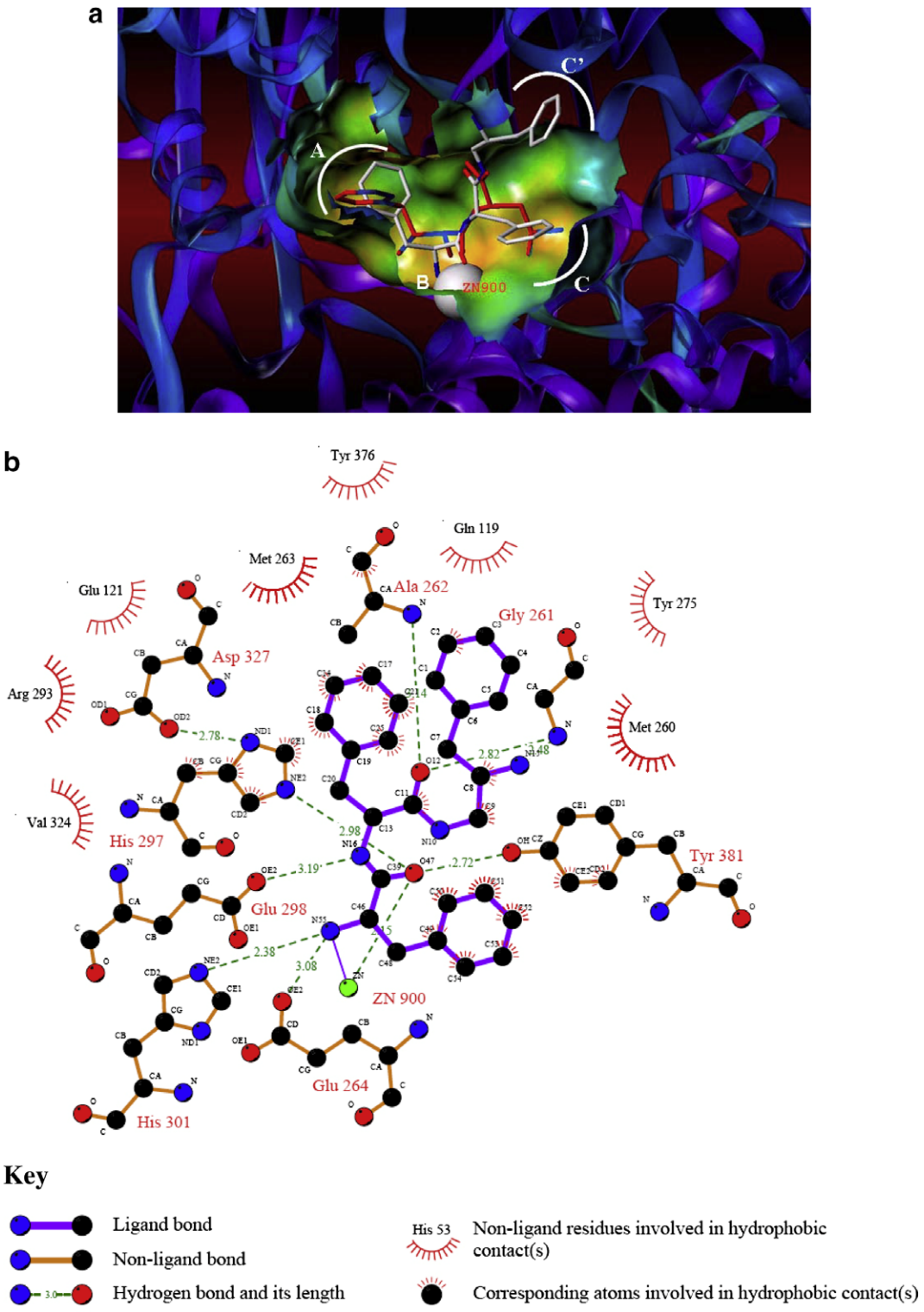


Figure 2. (a) The docking result of **12i** (Bestatin in the X-ray crystal is showed in red); (b) The docking result of **12i** showed by LIGPLOT.

alized with UV light, or iodine vapor. Proton NMR spectra were determined on a Brucker DRX spectrometer operating at 400 MHz for 1H, δ in parts per million and *J* in Hertz, using TMS as an internal standard. ESI-MS were determined on an API 4000 spectrometer. Measurements were made in D_2O, CD_3OD or DMSO-d_6 solutions. Melting points were determined on an electrothermal melting point apparatus and were uncorrected. Anhydrous reactions were carried out in over-dried glassware under a nitrogen atmosphere.

5.1.1. (2*R*)-2-[(*tert*-Butoxycarbonyl)amino]-3-phenylpropyl methanesulfonate (2)

Compound **1** (5 g, 20 mmol) was dissolved in anhydrous THF (20 mL) and Et_3N (3.03 g, 30 mmol) was added to the solution. A solution of methanesulfonyl chloride (3.4 g, 30 mmol) in anhydrous THF (10 mL) was added dropwise to the mixture. The reaction mixture was stirred for 4 h. The mixture was poured to the cold water and white solid was precipitated to give crude product

(*R*)-2-(*tert*-butoxycarbonyl)-3-phenylpropyl methanesulfonate (5.52 g yield 84.3%), which was of suitable purity to use directly in next reaction.

5.1.2. (2*R*)-2-[(*tert*-Butoxycarbonyl)amino]-3-phenylpropylamine (4)

A mixture of sulfonate **2** (10 g, 30 mmol) and NaN_3 (3.95 g, 60 mmol) in anhydrous DMF (80 mL) was stirred at 60 °C for 10 h. The mixture was poured to the cold water and extracted with ethyl acetate. The organic layer was washed with water and brine, dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by flash column chromatography to give on silica gel (Petroleum/EtOAc 10:1) to afford compound **3** (2*R*)-*tert*-butyl (2-azidomethyl-1-benzylethyl) carbamate.

The azide **3** (4 g, 0.015 mol) was hydrogenated on Mg (1.08 g, 0.045 mol) in MeOH at ordinary pressure and 0 °C for 1 h. MeOH was removed under reduced pressure and the residue was diluted with water. The aqueous solution was extracted efficiently with ether and the ether layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuum to give product **4** (2*R*)-2-[(*tert*-butoxycarbonyl)amino]-3-phenyl-propylamine. ESI-MS *m/z*: 251.1 $(M+H)^+$; ^1H NMR (MeOD) δ 1.41(s, 9H), 2.59–2.65 (J_1 = 6, J_2 = 12, dd, 1H), 2.71–2.81 (m, 3H), 3.79 (m, 1H), 7.18–7.32 (m, 5H).

5.1.3. 2-[(*tert*-Butoxycarbonyl)amino]-*N*-[(2*R*)-2-[(*tert*-butoxycarbonyl)amino]-3-phenylpropyl] acetamide (7a)

To a stirred solution of *tert*-butoxycarbonyl-2-amino-acetic acid (1.54 g, 8.80 mmol) and N-methylmorpholine (1.05 mL, 9.60 mmol) in THF (15 mL) was added isobutyl chloroformate (1.23 mL, 9.60 mmol) at −15 °C. The mixture was stirred for 30 min at the same temperature. A solution of compound **4** (2*R*)-2-[(*tert*-butoxycarbonyl)amino]-3-phenyl-propylamine (2.00 g, 8.00 mmol) in THF (20 mL) was added dropwise to the reaction mixture. The stirring was continued for 1 h at −15 °C and then remove the cooling bath. The reaction was continued for 4 h and the mixture was filtrated. After filtration, the filtrate was concentrated with a rotary evaporator. The residue was dissolved in EtOAc and washed with 5% $NaHCO_3$, 10% citric acid, and brine in turn. The EtOAc solution was dried over Na_2SO_4 and concentrated with a rotary evaporator to afford crude product. The crude product was recrystallized by EtOAc to afford pure title compound **7a**. Yield: 67.54%, mp = 103–105 °C. ESI-MS *m/z*: 408.3 (M+H); ^1H NMR (DMSO-d_6) δ 1.22 (s, 9H), 1.38 (s, 9H), 2.58–2.61 (m, 1H), 2.67–2.74 (J_1 = 5.4, J_2 = 13.8, dd, 1H), 3.01–3.07 (m, 1H), 3.16–3.19 (m, 1H), 3.50–3.56 (m, 2H), 3.66–3.68 (m, 1H) 7.17–7.27 (m, 5H).

5.1.4. 2-Amino-*N*-[(2*R*)-2-amino-3-phenylpropyl] acetamide hydrochloride (8a)

Compound **7a** (0.8 g, 1.96 mmol) was dissolved in 20 mL HCl–EtOAc (3 mol/L). After 30 min, the solvent was filtrated and the precipitate was washed with EtOAc to get 0.32 g of compound **8a** as a white solid. Yield: 78.66%, mp = 195.5–197.8 °C, $[\alpha]_D^{25}$ +26.8° (*c* 1, MeOH). ESI-MS *m/z*: 208.3 $(M+H)^+$; ^1H NMR (D_2O) δ *2.81–2.89* (J_1 = 6, J_2 = 15, dd, 1H), 2.94–3.01 (J_1 = 6, J_2 = 15, dd, 1H), 3.38–3.48 (J_1 = 4.5, J_2 = 15, dd, 1H), 3.49–3.56 (J_1 = 4.5, J_2 = 15, dd, 1H), 3.62–3.73 (m, 1H), 3.80 (s, 2H), 7.23–7.37 (m, 5H).

5.1.5. (2*S*)-2-Amino-4-methyl-*N*-[(2*R*)-2-amino-3-phenylpropyl]valeramide hydrochloride (8b)

Yield: 75.46%, mp = 165.5–168 °C, $[\alpha]_D^{25}$ +53.8° (*c* 1, MeOH). ESI-MS *m/z*: 264.0 $(M+H)^+$; ^1H NMR (D_2O) δ *0.86–0.87* (*J* = 3d, 6H),1.52–1.63 (m, 2H), 1.65–1.73 (m, 1H), 2.81–2.88 (J_1 = 6, J_2 = 15, dd, 1H), 2.94–3.01 (J_1 = 6, J_2 = 15, dd, 1H), 3.36–3.43 (J_1 = 6, J_2 = 15, dd, 1H), 3.49–3.56 (J_1 = 6, J_2 = 15, dd, 1H), 3.64–3.74 (m, 1H), 3.92–3.94 (*J* = 6, t, 1H), 7.22–7.36 (m, 5H).

5.1.6. (2*S*)-2-Amino-3-methyl-*N*-[(2*R*)-2-amino-3-phenylpropyl]propionamide hydrochl-oride (8c)

Yield: 72.45%, mp = 190–193.5 °C, $[\alpha]_D^{25}$ +55.0° (*c* 1, MeOH). ESI-MS *m/z*: 250.3 $(M+H)^+$; ^1H NMR (D_2O) δ *0.91–0.95* (*J* = 12, d, 6H), 2.12–2.17 (m, 1H), 1.65–1.73 (m, 1H), 2.80–2.88 (J_1 = 6, J_2 = 15, dd, 1H), 2.97–3.04 (J_1 = 6, J_2 = 15, dd, 1H), 3.31–3.40 (J_1 = 9, J_2 = 18, dd, 1H), 3.57–3.63 (J_1 = 6, J_2 = 15, dd, 1H), 3.60–3.63 (m, 1H), 3.71–3.73 (*J* = 6, d, 1H), 7.23–7.37 (m, 5H).

5.1.7. (2*S*)-2,6-Amino-*N*-[(2*R*)-2-amino-3-phenylpropyl]caproylamide hydrochloride (8d)

Yield: 78.48%, mp = 221.4–223 °C, $[\alpha]_D^{25}$ +61.6° (*c* 1, MeOH). ESI-MS *m/z*: 280.3 $(M+H)^+$; ^1H NMR (D_2O) δ *1.36–1.41* (m, 2H), 1.59–1.64 (m, 2H), 1.77–1.82 (m, 2H), 2.80–2.87 (J_1 = 6, J_2 = 15, dd, 1H), 2.87–2.90 (*J* = 9, t, 2H), 2.96–3.03 (J_1 = 6, J_2 = 15, dd, 1H), 3.34–3.40 (m, 1H), 3.55–3.62 (J_1 = 6, J_2 = 15, dd, 1H), 3.62–3.68 (J_1 = 6, J_2 = 15, dd, 1H), 3.89–3.93 (J_1 = 12, t, 1H), 7.23–7.37 (m, 5H).

5.1.8. (2*S*)-2-Amino-4-methylthio-*N*-[(2*R*)-2-amino-3-phenylpropyl]butyramide hydrochloride (8e)

Yield: 72.41%, mp = 82–84.5 °C, $[\alpha]_D^{25}$ +19.2° (*c* 1, MeOH). ESI-MS *m/z*: 283.4 $(M+H)^+$; ^1H NMR (D_2O) δ 2.01 (s, 3H), 2.05–2.09 (m, 2H), 2.50–2.55 (*J* = 15, t, 2H), 2.80–2.87 (J_1 = 6, J_2 = 15, dd, 1H), 2.92–3.00 (J_1 = 6, J_2 = 15, dd, 1H), 3.47–3.53 (m, 1H), 3.55–3.610 (J_1 = 6, J_2 = 12, dd, 1H), 3.62–3.69 (J_1 = 6, J_2=12, dd, 1H), 3.62–3.66 (*J* = 12, t, 1H), 7.22–7.37 (m, 5H).

5.1.9. (2*S*)-2-Amino-3-hydroxyl-*N*-[(2*R*)-2-amino-3-phenylpropyl]propionamide hydrochloride (8f)

Yield: 78.61%, mp = 111–114 °C, $[\alpha]_D^{25}$ +19.5° (*c* 1, MeOH). ESI-MS *m/z*: 238.4$(M+H)^+$; ^1H NMR (D_2O) δ *2.80–2.88* (J_1 = 6, J_2 = 13.5, dd, 1H), 2.96–3.03 (J_1 = 6, J_2 = 12.6, dd, 1H), 3.47–3.49 (*J* = 6, d, 2H), 3.64–3.69 (m, 1H), 3.85–3.90 (m, 2H), 4.03–4.06 (*J* = 9, t, 1H), 7.23–7.37 (m, 5H).

5.1.10. (2*S*)-2,5-Amino-*N*-[(2*R*)-2-amino-3-phenylpropyl]-valeramide hydrochloride (8g)

Yield: 68.73%, mp = 130–133.3 °C, $[\alpha]_D^{25}$ +16.8° (*c* 1, MeOH). ESI-MS *m/z*: 265.2 $(M+H)^+$; ^1H NMR (D_2O) δ *1.64–1.72* (m, 2H), 1.82–1.88 (m, 2H), 2.81–2.88 (J_1 = 6.6, J_2 = 15, dd, 1H), 2.89–2.96 (J_1 = 6, J_2 = 14.4, dd, 1H), 3.01–3.03 (m, 2H), 3.34–3.40 (m, 1H), 3.57–3.63 (m, 2H), 3.93–3.99 (*J* = 15, t, 1H), 7.23–7.36 (m, 5H).

5.1.11. (2*S*)-2-Amino-*N*-[(2*R*)-2-amino-3-phenylpropyl]-propionamide hydrochloride (8h)

Yield: 67.54%, mp = 161–163.5 °C, $[\alpha]_D^{25}$ +28.4° (*c* 1, MeOH). ESI-MS *m/z*: 222.4$(M+H)^+$; ^1H NMR (D_2O) δ *1.42–1.46* (*J* = 12, d, 3H), 2.82–2.89 (J_1 = 6, J_2 = 14.4, dd, 1H), 2.96–3.03 (J_1 = 5.4, J_2 = 15, dd, 1H), 3.41–3.50 (m, 2H), 3.64–3.71 (m, 1H), 4.01–4.08 (m, 1H), 7.24–7.39 (m, 5H).

5.1.12. *N*-[(2*R*)-2-Amino-3-phenylpropyl]-L-glutamamide hydrochloride (8i)

Yield: 70.53%, mp = 167–171 °C, $[\alpha]_D^{25}$ +24.6° (*c* 1, MeOH). ESI-MS *m/z*: 279.4 $(M+H)^+$; ^+H NMR (D_2O) δ *1.98–2.07* (m, 2H), 2.35–2.40 (*J* = 15, t, 2H), 2.83–2.89 (J_1 = 6.3, J_2 = 14.1, dd, 1H), 2.96–3.03 (J_1 = 6, J_2 = 14.7, dd, 1H), 3.38–3.45 (J_1 = 4.8, J_2 = 14.4, dd, 1H), 3.54–3.61 (J_1 = 4.5, J_2 = 14.7, dd, 1H), 3.63–3.67 (*J* = 12, t, 1H), 3.95–3.99 (m, 1H), 7.24–7.38 (m, 5H).

5.1.13. (2*S*)-2-Amino-3-thiol-*N*-[(2*R*)-2-amino-3-phenylpropyl]propionamide hydrochloride (8j)

Yield: 61.28%, mp = 156–158 °C, $[\alpha]_D^{25}$ +30.3° (*c* 1, MeOH). ESI-MS *m/z*: 254.3$(M+H)^+$; ^1H NMR (D_2O) δ *2.83–2.89* (J_1 = 5.4, J_2 = 13.2, dd, 1H), 2.92–2.99 (J_1 = 5.1, J_2 = 12.6, dd, 1H), 3.08–3.15 (J_1 = 6, J_2 = 13.5, dd, 1H), 3.27–3.32 (J_1 = 5.7, J_2 = 12.6, dd, 1H),

3.42–3.44 (J = 5.7, d, 2H), 3.47–3.52 (m, 1H), 4.10–4.12 (J = 6.3, t, 1H), 7.25–7.37 (m, 5H).

5.1.14. (3S)-3-[(tert-Butoxycarbonyl)amino]-N-[(2R)-2-[(tert-butoxycarbonyl)amino]-3-phenylpropyl]propionamide hydrochloride (11a)

N-(*tert*-Butoxycarbonyl)-β-alanine (2.00 g, 10.58 mmol) was dissolved in anhydrous CH_2Cl_2 (30 mL). The solution of oxalyl chloride (2.01 g, 15.87 mmol) in CH_2Cl_2 was added dropwise surrounded with ice bath. After 4 h, CH_2Cl_2 was removed in vacuum to afford compound 10 (2.07 g).

Compound **4** (2.00 g, 8.00 mmol) was dissolved in anhydrous THF (40 mL) and Et_3 N (1.33 mL). After 10 mL THF with 1.99 g compound **13** was added dropwise at 0 °C and stirred for 4 h, THF was removed under reduced pressure and the residue was diluted with EtOAc. The organic phase was washed with 5% $NaHCO_3$, 10% citric acid, and brine in turn. The EtOAc solution was dried over Na_2SO_4 and concentrated with a rotary evaporator to afford crude product. The crude product was recrystallized by EtOAc to afford pure title compound **11a**. Yield: 63.82%, mp = 121–123 °C. ESI-MS *m/z*: 422.3 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ 1.31 (s, 9H), 1.36 (s, 9H), 2.21–2.26 (m, 2H), 2.53–2.61 (J_1 = 5.1, J_2 = 13.5, dd, 1H), 3.01–3.07 (m, 1H), 2.67–2.73 (J_1 = 5.4, J_2 = 13.5, dd, 1H), 3.11–3.15 (m, 4H), 3.68–3.70 (m, 1H) 7.17–7.28 (m, 5H).

5.1.15. (3S)-3-Amino-N-[(2R)-2-amino-3-phenylpropyl]propionamide hydrochloride (12a)

Compound **11a (0.8 g,** 1.96 mmol) was dissolved in 20 mL HCl–EtOAc (3 mol/L). After 30 min, the solvent was filtrated and the precipitate was washed with EtOAc to get 0.39 g of compound 12a as a white solid. Yield: 85.68%, mp = 179–181 °C, $[\alpha]_D^{25}$ +54.9° (*c* 1, MeOH). ESI-MS *m/z*: 222.3 $(M+H)^+$; ^{1}H NMR (D_2O) δ *2.57–2.62* (J = 15, t, 2H), 2.82–2.89 (J_1 = 6, J_2 = 15, dd, 1H), 2.93–3.00 (J_1 = 6, J_2 = 15, dd, 1H), 3.13–3.18 (J = 15, t, 2H), 3.32–3.39 (J_1 = 6, J_2 = 15, dd, 1H), 3.44–3.50 (J_1 = 6, J_2 = 15, dd, 1H), 3.62–3.69 (m, 1H), 7.23–7.37 (m, 5H).

5.1.16. 6-Amino-N-[(2R)-2-amino-3-phenylpropyl]caproylamide hydrochloride (12b)

Yield: 65.42%, mp = 135–137.3 °C, $[\alpha]_D^{25}$ +46.2° (*c* 1, MeOH). ESI-MS *m/z*: 264.2 $(M+H)^+$; ^{1}H NMR (D_2O) δ *1.24–1.32* (m, 2H), 1.45–1.62 (m, 4H), 2.15–2.20 (J = 15, t, 2H), 2.80–2.86 (J_1 = 3, J_2 = 12, dd,1H), 2.86–2.91 (J = 15, t, 2H), 2.94–3.01 (J_1 = 3, J_2 = 12, dd, 1H), 3.31–3.38 (J_1 = 3, J_2 = 12, dd, 1H), 3.41–3.48 (J_1 = 3, J_2 = 12, dd, 1H), 3.62–3.68 (m, 1H), 7.22–7.37 (m, 5H).

5.1.17. N-[(2R)-2-Amino-3-phenylpropyl]-3-phenylpropanamide hydrochloride (12c)

Yield: 77.37%, mp = 184.8–188.3 °C, $[\alpha]_D^{25}$ +23.5° (*c* 1, MeOH). ESI-MS *m/z*: 283.3 $(M+H)^+$; ^{1}H NMR (D_2O) δ *2.46–2.51* (J = 14.4, t, 2H), 2.55–2.62 (J_1 = 6, J_2 = 14.4, dd, 1H), 2.63–2.70 (J_1 = 6.3, J_2 = 15, dd, 1H), 2.79–2.84 (J = 15, t, 2H), 3.17–3.24 (J_1 = 6, J_2 = 15, dd, 1H), 3.24–3.31 (J_1 = 6, J_2 = 14.4, dd, 1H), 3.38–3.45 (m, 1H), 7.08–7.18 (m, 5H), 7.22–7.33 (m, 5H).

5.1.18. 1 (2E)-N-[(2R)-2-Amino-3-phenylpropyl]-3-phenylacrylamide hydrochloride (12d)

Yield: 67.89%, mp = 191–195 °C, $[\alpha]_D^{25}$ +16.8° (*c* 1, MeOH). ESI-MS *m/z*: 281.3 $(M+H)^+$; ^{1}H NMR (MeOD) δ *3.00–3.03* (J = 9, d, 2H), 3.49–3.56 (J_1 = 3.9, J_2 = 9.6, dd, 1H), 3.58–3.65 (J_1 = 3.6, J_2 = 8.4, dd, 1H), 3.67–3.73 (m, 1H), 7.68–7.73 (J = 15, d, 1H), 7.31–7.63 (m, 11H).

5.1.19. N-[(2R)-2-Amino-3-phenylpropyl]-2-phenylacetamide hydrochloride (12e)

Yield: 73.65%, mp = 67–69.5 °C, $[\alpha]_D^{25}$ +13.0° (*c* 1, MeOH). ESI-MS *m/z*: 269.5 $(M+H)^+$; ^{1}H NMR (MeOD) δ *2.92–2.95* (d, 2H), 3.32–3.34(J = 6, d, 1H) 3.40–3.42 (J = 6, d, 1H), 3.59–3.62 (m, 3H), 7.24–7.39 (m, 10H).

5.1.20. N-[(2R)-2-Amino-3-phenylpropyl]-4-phenylbutanamide hydrochloride (12f)

Yield: 71.34%, mp = 102–104.5 °C, $[\alpha]_D^{25}$ +48.7° (*c* 1, MeOH). ESI-MS *m/z*: 297.5 $(M+H)^+$; ^{1}H NMR (D_2O) δ *1.73–1.90* (m, 2H), 2.11–2.15 (J = 12, t, 2H), 2.50–2.55 (J = 12, t, 2H), 2.77–2.84 (J_1 = 6.3, J_2 = 14.1, dd, 1H), 2.86–2.93 (J_1 = 6.6, J_2 = 14.1, dd, 1H), 3.21–3.28 (J_1 = 6, J_2 = 14.7, dd, 1H), 3.32–3.40 (J_1 = 6, J_2 = 14.7, dd, 1H), 3.54–3.58 (m, 1H), 7.15–7.34 (m, 10H).

5.1.21. N-[(2R)-2-Amino-3-phenylpropyl]-2-{2-[(2,6-dichlorophenyl)amino]phenyl} acetamide hydrochloride (12g)

Yield: 76.83%, mp = 85–87 °C, $[\alpha]_D^{25}$ +20.0° (*c* 1, MeOH). ESI-MS *m/z*: 428.5 $(M+H)^+$; ^{1}H NMR (D_2O) δ *2.81–2.88* (J_1 = 3, J_2 = 15, dd, 1H), 2.95–3.01 (J_1 = 3, J_2 = 12, dd, 1H), 3.22–3.30 (m, 2H), 3.67 (s, 2H), 4.00–4.06 (m, 1H), 6.27–6.30 (J = 6, d, 1H), 6.83–6.87 (J = 12, 1H), 7.02–7.07 (J = 15, t, 1H), 7.17–7.19 (J = 6, d, 1H), 7.23–7.30 (m, 6H), 7.50–7.53 (J = 9, d, 2H).

5.1.22. (2E)-N-[(2R)-2-Amino-3-phenylpropyl]-3-(3,4-hydroxy)-acrylamide hydrochloride (12h)

Yield: 55.37%, mp = 143–145 °C, $[\alpha]_D^{25}$ +21.6° (*c* 1, MeOH). ESI-MS *m/z*: 313.4 $(M+H)^+$; ^{1}H NMR (D_2O) δ *2.82–2.98* (m, 2H), 2.98–3.05 (m, 2H), 3.72–3.74 (m, 1H), 6.37–6.42 (J = 15, d, 1H), 6.74–6.79 (J = 15, d, 1H), 7.21–7.38 (m, 8H).

5.1.23. L-Phenylalanyl-N-[(2R)-2-amino-3-phenylpropyl]-L-phenylalaninamide hydrochloride (12i)

Yield: 69.54%, mp = 90–93 °C, $[\alpha]_D^{25}$ +40.9° (*c* 1, MeOH). ESI-MS *m/z*: 445.5 $(M+H)^+$; ^{1}H NMR (D_2O) δ *2.75–2.95* (m, 5H), 3.10–3.16 (m, 2H), 3.28–3.34 (m, 2H), 4.00–4.06 (J_1 = 4.5, J_2 = 12, dd, 1H), 4.52–4.54 (m, 1H), 7.27–7.31 (m, 15H).

5.1.24. N-[(2R)-2-Amino-3-phenylpropyl]-3,5-dinitrobenzamide hydrochloride (12j)

Yield: 49.48%, mp = 205–207 °C, $[\alpha]_D^{25}$ +39.1° (*c* 1, MeOH). ESI-MS *m/z*: 345.1 $(M+H)^+$; ^{1}H NMR (D_2O) δ *2.85–2.92* (J_1 = 5.4, J_2 = 13.8, dd, 1H), 2.99–3.06 (m, 1H), 3.40–3.47 (m, 1H), 3.55–3.60 (m, 2H), 7.26–7.36 (m, 5H), 8.97 (s, 1H), 9.03 (s, 2H).

5.2. Biological materials and methods

5.2.1. In vitro APN assay

IC_{50} values against APN were determined as previously described and using L-Leu-*p*-nitroanilide as substrate and Microsomal aminopeptidase from Porcine Kidney Microsomes (Sigma) as the enzyme in 50 mM PBS, pH 7.2, at 37 °C. The hydrolysis of the substrate was monitored by following the change in the absorbance measured at 405 nm with the UV–vis spectrophotometer Pharmacia LKB, Biochrom 4060. All solutions of inhibitors were prepared in the assay buffer, and pH was adjusted to 7.5 by the addition of 0.1 M HCl or 0.1 M NaOH. All inhibitors were preincubated with APN for 30 min at room temperature. The assay mixture, which contained the inhibitor solution (concentration dependent on the inhibitor), the enzyme solution (4 μg/mL final concentration), and the assay buffer, was adjusted to 200 μL.

5.2.2. In vitro MMP-2 assay

Gelatinase A (MMP-2) and TNBS were purchased from Sigma, and the substance was synthesized as described by Vijaykumar et al. The gelatinase, substance, and inhibitor were dissolved in so-

dium borate (pH 8.5, 50 mmol/L) and incubated for 30 min at 37 °C, and then 0.03% TNBS was added and incubated for another 20 min, the resulting solution was detected under 450 nm wavelength to gain absorption.

5.2.3. MTT assay

HL-60 cell was grown in RPMI1640 medium containing 10% FBS at 37 °C in 5% CO_2 humidified incubator. Cell proliferation was determined by the MTT (3-[4,5-dimethyl-2-thiazolyl]-2.5-diphenyl-2H-tetrazolium bromide) assay. Briefly, cells were plated in a 96-well plate at 10,000 cells per well, cultured for 4 h in complete growth medium, then treated with 2000, 1000, 500, 250, and 125 μg/mL of compounds for 48 h. 0.5% MTT solution was added to each well. After further incubation for 4 h, formazan formed from MTT was extracted by adding DMSO and mixing for 15 min. Optical density was read with an ELISA reader at 570 nm.

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