Design, Synthesis and Preliminary Activity Evaluation of Novel L-Lysine Derivatives as Aminopeptidase N/CD13 Inhibitors

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Abstract: A novel class of L-lysine derivatives as aminopeptidase N (APN) inhibitors was designed and synthesized. Activity evaluation showed that compound **C7** (IC_{50} = 9.6 ± 1.3 μM) and **C20** (IC_{50} = 13.6 ± 1.9 μM) were equivalent to the positive control Bestatin (IC_{50} = 11.3 ± 1.6 μM).

Keywords: Lysine derivatives, Synthesis, APN; Inhibitor.

1. INTRODUCTION

Belonging to M1 aminopeptidase family, aminopeptidase N (APN) is a Zn^{2+} dependent exopeptidase [1], which is expressed by myeloid, monocytes, epithelial cells of the intestine and kidney, fibroblasts and endothelial cells, and overexpressed on tumor cells [2]. APN plays a critical role in physiological modification and protein degradation as well as in the process of tumor cell metastasis [3-6].

Numerous natural and synthetic small molecule inhibitors against APN have been reported in the literatures since 1976 [7-16]. Our group also has reported various series of APN inhibitors, such as 3-galloylamido-*N*'-substituted 2,6-piperidinedione-*N*-acetamide peptidomimetics [17], L-glutamine derivatives[18], AHPA (β-Amino-α-hydroxyl-phenylbutanoic acid) derivates and L-arginine derivatives [19, 20].

In 2006, the structure of the complex of bestatin and aminopeptidase N (EC 3.4.11.2) from *Escherichia coli* (ePepN) was reported (PDB ID: 2DQM, Resolution: 1.60Å) [21]. Then, the structure of the complex of L-lysine and ePepN was reported in 2008 (PDB ID: 3B2X, Resolution: 1.50Å) [22]. Examining the active site of these complexes by Sybyl, we found that bestatin and L-lysine have different binding modes to APN. Bestatin can occupy hydrophobic pocket A and pocket C concurrently while binding with zinc ion, but L-lysine only occupies pocket A while binding with zinc ion (Fig. **1**).

We also reported compounds **12i** (L-phenylalanyl-*N*-[(2*R*)-2-amino-3-phenylpropyl]-L-phenylalaninamide) and **13b** ((*S*)-2-((*S*)-2-((2*R*,3*S*)-2-Amino-3-hydroxy-3-(4-nitrophenyl)propanamido)-3-phenylpropanamido)-3-phenylpropanoic acid), both showing high APN inhibitory activities [23, 24]. An *in silico* docking study disclosed that these compounds are proposed to bind the zinc ion with the carbonyl group and near amino group in the active site. These interesting results reminded us that this kind of zinc ion binding groups could also be used to develop novel APN inhibitors. Moreover, free amino group as hydrogen bond site may contribute to water-solubility of the compounds.

In order to occupy pockets A and C concurrently, we modified L-lysine with the same zinc binding group mentioned above to develop novel APN inhibitors as depicted in the following scheme (Fig. **1**): R_1 is an aromatic ring, linked to α-COOH of alkyl chain by amide bond. N^6-amino group was selectively protected by carbobenzoxy (Cbz) moiety. N^2-amino group was close to α-carbonyl group for chelating with the zinc ion.

2. CHEMISTRY

The synthetic route of target compounds is shown in Scheme **1**. The N^6-amino group was selectively Cbz-protected, followed by the Boc-protection of the N^2-amino group to provide compound **8** N^6-[(benzyloxy)carbonyl]-N^2-(*tert*-butoxycarbonyl)-L-lysine. Then, 20 different aromatic amines reacted with α-COOH forming an amide bond. Finally, the N^2-Boc protecting group was cleaved in HCl-EtOAc (3 mol/L) to obtain the target compounds[1].

3. RESULTS

The target compounds were assayed for the inhibitory activities on APN[2]. The biological results (Table **1**) exhibited

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[1] All new compounds **C1-20** provided acceptable HR-MS and ^{1}H-NMR spectra that exhibit no discernible impurities. For example, compound **C7**: White solid, yield 73.5 %, mp 193-195 ℃. HR-MS (ESI) m/z calcd for $C_{19}H_{25}N_4O_3^+$ $[M+H]^+$ 357.1921. Found: 357.1929; ^{1}H-NMR (600 MHz, DMSO-d_6) δ 11.43 (s, 1H), δ 8.63 (brs, 2H), 8.58 (m, 1H), δ 8.39 (m, 1H), δ 8.16 (s, 1H), δ 8.05 (m, 1H), δ 7.94 (m, 1H), δ 7.31 (m, 5H), δ 4.97 (s, 2H), δ 4.11 (m, 1H), δ 2.75 (m, 2H), δ 1.86 (m, 2H), δ 1.63(m, 2H), δ 1.40 (m, 2H).

[2] APN inhibition assay: IC_{50} values against APN were determined as 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 ℃. The

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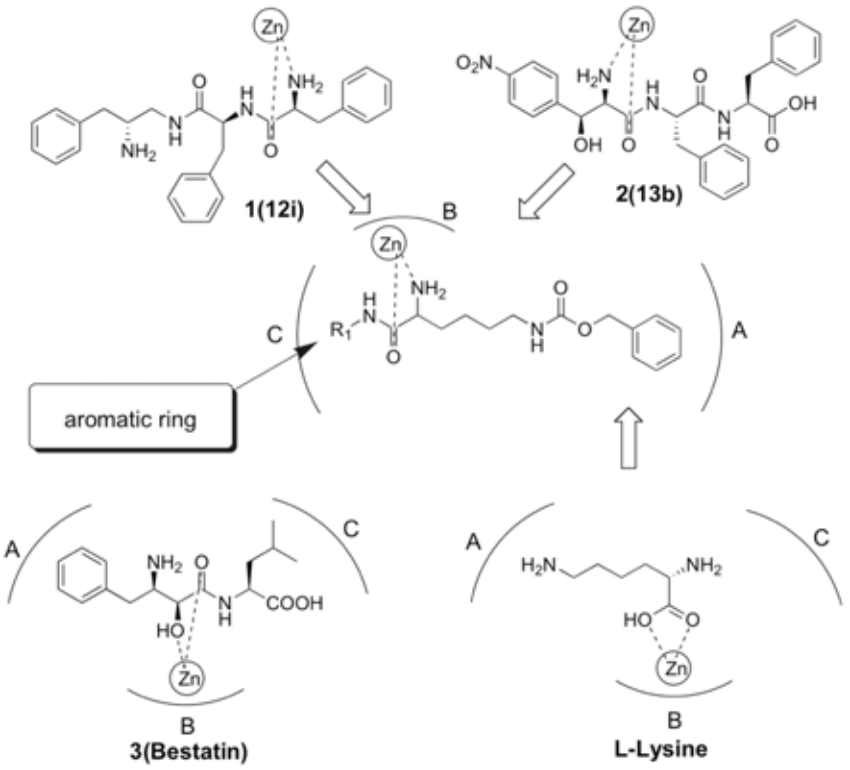
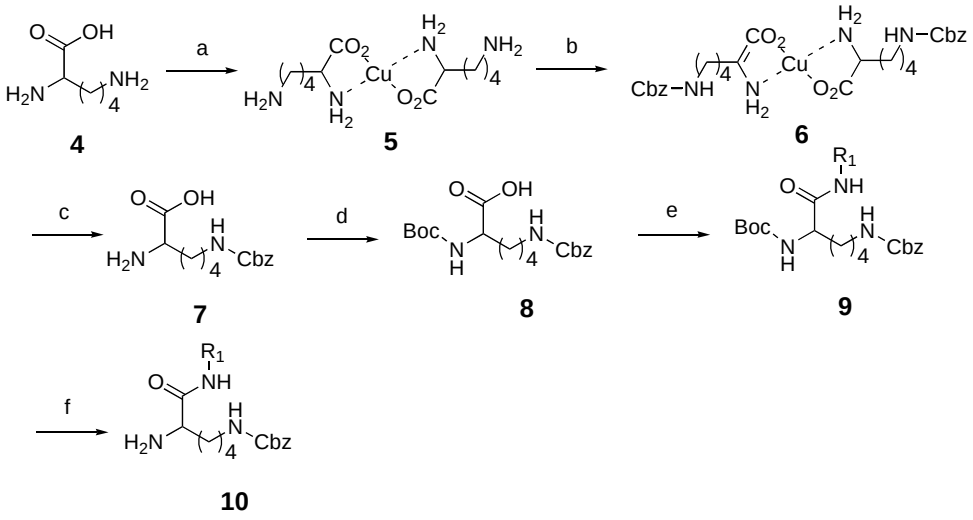


Figure 1. The design of target compounds.



Scheme 1. Reagents and conditions: (a) $CuCO_3Cu(OH)_2$, 1.2 N HCl, H_2O, 80-90 ℃, 2 h; (b) $NaHCO_3$, CbzCl, 25 ℃, 12 h; (c) EDTA SS, 25 ℃, 24 h; (d) $(Boc)_2O$, THF, 1.0 N NaOH, 0 ℃, 12 h; (e) R_1NH_2, Et_3N, TBTU, CH_2Cl_2, 25 ℃, 5 h; (f) HCl–EtOAc, 24 h.

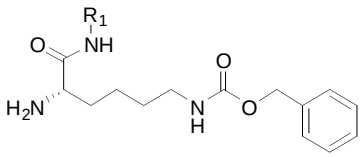
that the activities of compound **C7** (IC_{50} = 9.6 ± 1.3 μM) and **C20** (IC_{50} = 13.6 ± 1.9 μM) were equivalent to that of positive control bestatin (IC_{50} = 11.3 ± 1.6 μM).

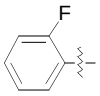
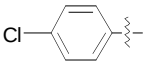
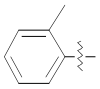
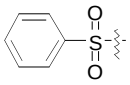
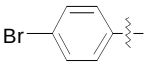
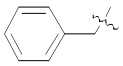
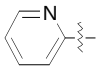
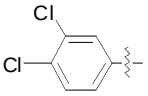
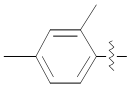
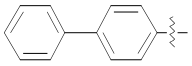
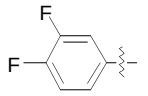
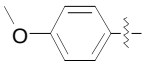
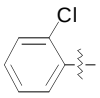
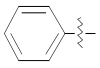
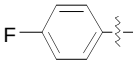
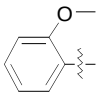
Additionally, the effects of **C7** on proliferation of six tumor cells compared with bestatin are shown in Table **2**. Cell viability was assessed by MTT method[3]. Compound **C7** showed better potency of anti-proliferation than bestatin to HL-60 and PLC cells. But for ES-2 cell, they were equivalent.

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 compounds 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 compounds 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.

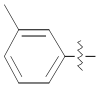
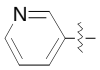
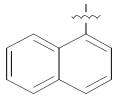
[3] MTT assay: For example, HL-60 cell was grown in RPMI1640 medium containing 10 % FBS at 37 ℃ in 5 % CO_2 humidified incubator. Cell proliferation was determined by the MTT (3-[4,5-dimethyl-2-thiazolyl]-2.5-diphenyl-2H-tetrazolium bromide) assay. Briefly, cells were plated in a 96-well plate at 10,000 cells per well, cultured for 4 h in complete growth medium, then treated with 2000, 1000, 500, 250 and 125 lg/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.

Table 1. The Inhibitory Activities of Target Compounds Against APN



Compound	R_1	IC_{50}*(μM)
C1		212.1±21.7
C2		91.3±10.9
C3		410.5±44.0
C4		>1000
C5		361.0±44.3
C6		35.6±4.6
C7		9.6±1.3
C8		810.4±84.7
C9		277.5±54.0
C10		>1000
C11		783.8±86.4
C12		185.1±33.9
C13		260.5±51.3
C14		475.9±57.2
C15		460.6±43.0
C16		513.3±55.5

(Table 1) contd…..

Compound	R_1	IC_{50}*(μM)
C17		632.4±89.2
C18		215.8±31.0
C19		224.9±29.9
C20		13.6±1.9
Bestatin		11.3±1.6

*Each value represents the mean of three experiments, standard deviation is given.

Table 2. The Inhibitory Activities of Compound C7 and Bestatin Against Tumor Cell Lines

Compound	IC_{50}*(mM)					
	HL-60	**ES-2**	**K562**	**A549**	**H7402**	**PLC**
C7	1.17±0.22	0.98±0.21	0.40±0.17	0.63±0.23	0.98±0.16	0.81±0.11
Bestatin	1.42±0.31	0.87±0.11	0.28±0.09	0.07±0.05	0.63±0.16	5.42±0.91

*Each value represents the mean of three experiments, standard deviation is given.

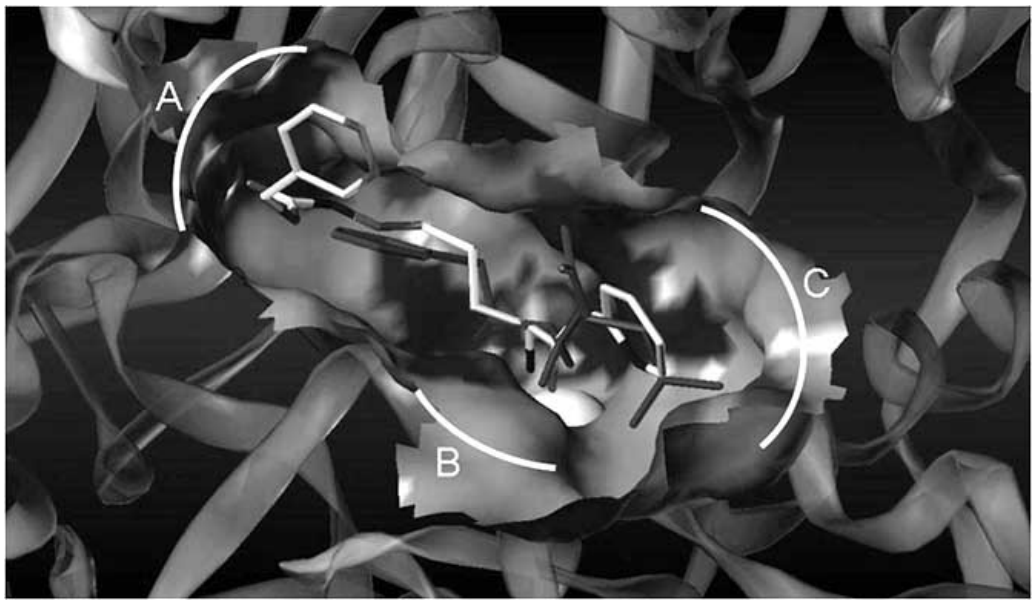


Figure 2. The docking result of compound C7. Bestatin in the X-ray crystal structure is showed in dark color.

In order to investigate the interaction of our target compounds with APN, the representative compound **C7** was constructed with Sybyl/Sketch module and optimized using Powell's method with the Tripos force field with convergence criterion set at 0.05 Kcal/mol•Å, and assigned with Gasteiger-Hückel method. The docking study was performed using Sybyl/FlexX module, the residues in a radius of 7.0 Å around Bestatin in the co-crystal structure (PDB code: 2DQM) were selected as the active site. Other docking parameters implied in the program were kept at default. The docking studies proposed that the carbonyl group and amino group of compound **C7** bind to the zinc ion and form a bi-

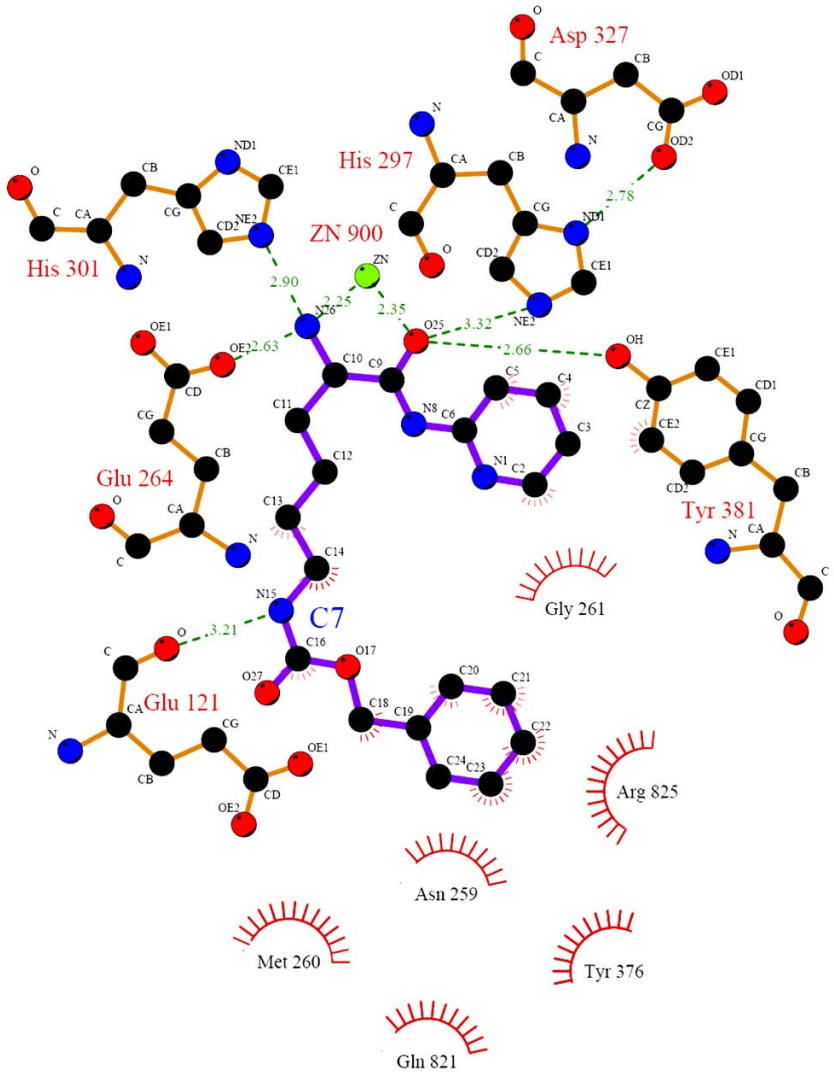


Figure 3. The docking result of compound C7 as suggested by LIGPLOT.

dentate chelate in the active pocket with distance of 2.3 Å and 2.4 Å, respectively (Figs. **2**,**3**). Furthermore, the distance of His[297] and His[301] both binding to the zinc ion was 2.1 Å.

In addition, the N^6-Cbz could occupy the pocket A and R_1 group for interacting with the pocket C. Compound **C7** could form hydrogen bonds with His[297] (< 3.3 Å) and His[301] (< 2.9 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 [25].

4. DISCUSSION

As shown in the results, 2 or 4-substitution of R_1 could improve the inhibitory effect weakly by comparing **C1**, **C2**, **C3**, **C5**, **C9**, **C12**, **C13** to **C14** except for **C10** and **C16**, which demonstrated that steric effect dominated the substitution result. Especially, 4-chloro substitution exhibited better potency than the others. 3-substitution of R_1 would decrease the inhibitory effect by comparing **C8**, **C11**, **C17** to **C14**.

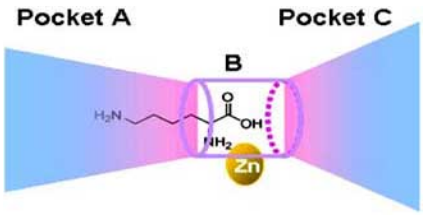


Figure 4. The active site of the complex of L-lysine and ePepN.

By examining the active site of the complex between L-lysine and ePepN (PDB ID: 3B2X, Fig. **4**), we inferred that our target compounds might interact with APN by three steps as showed in Fig. (**5**).

The association of the heterocycle N and the amide group N in the same plane of R_1 could be treated as a potent bidentate ZBG, both for **C7** or **C20**, which would increase the possibility to bind with the zinc ion when interacting with APN in step 2 as a transition state. As a result, it might in-

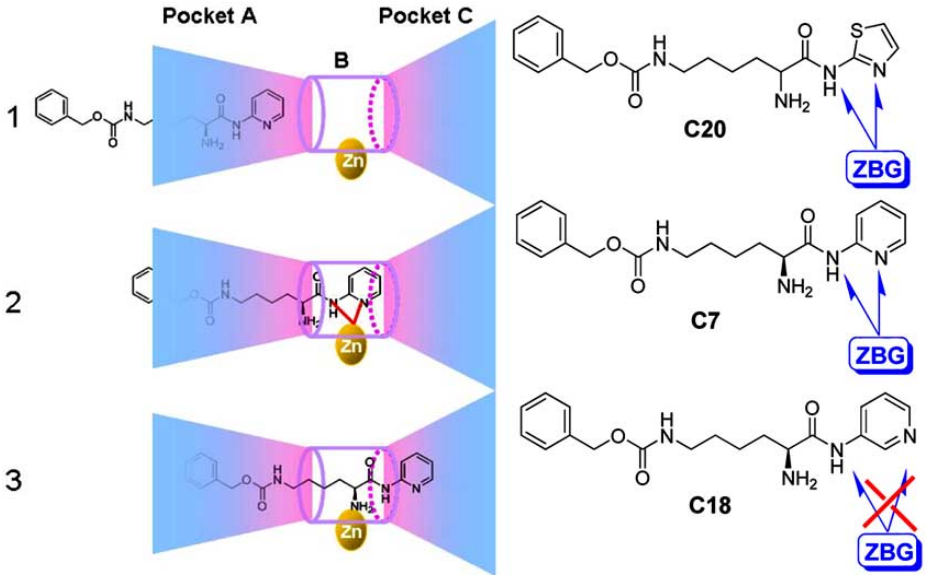


Figure 5. The selectivity of the active site in APN to compounds C7, C18 and C20.

crease the inhibitory activities of compounds **C7** and **C20.** But for compound **C18**, the heterocycle N might be too far away from the amide group N to associate as a potent bidentate ZBG.

However, the poor inhibitory activity of compound **C4** might be caused by the formation of an intramolecular hydrogen bond between the free amino group and its neighboring sulfonyl group, which would repress the ZBG to bind with the zinc ion in the active site of APN.

The rotatable C-N bond between benzene and amide bond of compound **C6** might make it interact with APN in a lower energy conformation comparing to the other compounds. This might be the reason why compound **C6** exhibited better potency than compound **C14**. The compound **12i** mentioned above worked similarly.

CONCLUSIONS

In summary, we developed a class of novel L-Lysine derivatives as potential APN inhibitors. Compounds **C7** and **C20** exhibited similar inhibitory activities compared with bestatin. Therefore, they could be lead compounds for us to develop new L-lysine derivatives as APN inhibitors.

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