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Original article

Novel cyclic-imide peptidomimetics as aminopeptidase N inhibitors. Design, chemistry and activity evaluation. Part I

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ABSTRACT

A series of aminopeptidase inhibitors with cyclic-imide scaffold are described. The biological characterization for the piperidinedione analogues revealed that most compounds displayed high inhibitory activity against APN. Among which **4l** and **6** showed potent inhibition against APN with the IC_{50} value of 5.2 μM and 3.1 μM, respectively. In addition, **6** also displayed good activity in HL-60 cell assay and in vivo anti-metastasis assay. This interesting activity profile may also guide the design of new, specific inhibitors of target mammalian aminopeptidases with 'one-zinc' active site.

1. Introduction

Aminopeptidase N (APN, EC3.4.11.2, CD13), an ectopeptidase expressed widely by monocytes, myeloid, epithelial cells of the intestine and kidney, fibroblasts, endothelial cells and tumor cells, is of significant biological and medical importance because of its key role in protein modification, activation, and degradation as well as in the metabolism of biologically active peptides in tumor metastasis and leukemia [1–4]. The design and synthesis of inhibitors for aminopeptidase N may result in potential therapeutic agents.

Since 1976, several excellent reviews on natural and synthetic small molecule inhibitors of APN have been published [5,6]. Among 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 [7].

In recent years, the availability of X-ray crystal structures for APN and complexes with various inhibitors has been investigated [8–10]. Our group had reported some new APN inhibitors, such as L-lysine derivates [11], L-iso-glutamine derivatives [12], AHPA (b-amino-a-hydroxyl-phenylbutanoic acid) derivates [13] and 3-phenylpropane-1,2-diamine derivates [14,15]. Both Yoshimoto's and Matthews's groups had reported the binding site and catalytic domain of APN based on the co-crystal complex of *Escherichia coli* APN and bestatin [8,10]. The binding site of the APN with bestatin has several characteristics: part I is a hydrophobic pocket interacting with phenyl group of bestatin; part II is one zinc ion which can interact with the zinc binding group in the structure of inhibitors (for bestatin, they are 2-hydroxyl group and 1-carbonyl group of amide); part III is another hydrophobic pocket in deeper cavity which can be divided into two subsites [5,11,16].

In our previous work, we have reported a series of L-iso-glutamine derivatives [12]. The biological characterization revealed that most compounds displayed good inhibitory activity against APN. Herein, in order to find better APN inhibitors, we modified the structure as the following: the γ-carboxyl group and the nitrogen of the amide were cyclized to explore new scaffold of cyclic-imide; the R_2 group can be OH, OCH_3 and NHOH or some amino acid derivatives to form tri-peptidomimetics, Fig. 1. To improve the bioactivity, the gallic acid moiety was kept because of its anti-tumor and anti-oxidative activities [17,18] and R_1 group can be either H or CH_3. The in vitro inhibitory activity was measured against APN enzyme. Additionally, potent compounds are also evaluated on HL-60 cell line and in vivo anti-metastasis assay.

2. Chemistry

The critical intermediate **3** was synthesized following the procedures as shown in Scheme 1. The starting material (*S*)-2-(3,4,5-trimethoxybenzamido)pentanedioic acid (**1**) was

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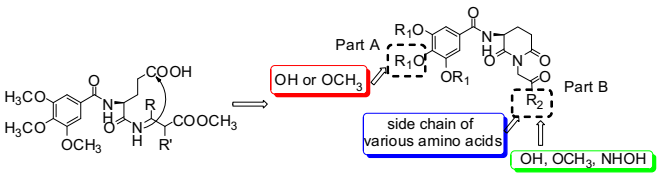


Fig. 1. Strategy for the design of cyclic-imide derivatives.

prepared from L-Glutamine following Reference [12]. The two carboxyl groups of L-glutamine were cyclized with acetic acid anhydride as dehydration reagent at 55–60 °C to afford compound **2**. Compound **3** was obtained using one-pot reaction from compound **2** and glycine, during which process microwave heating was applied. All the target compounds **4a–r** were obtained in good yield by condensation of carboxyl group of **3** and appropriate amino acid derivatives with EDCI as the coupling reagent.

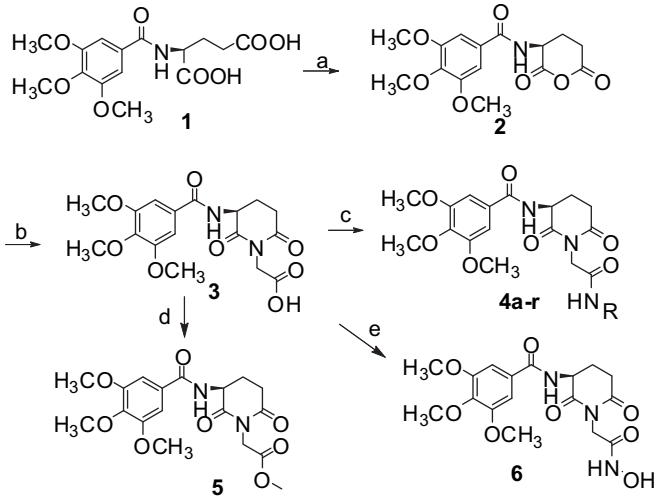
Additionally, the carboxyl group of compound **3** was converted to its methyl ester in the existence of $SOCl_2$ and CH_3OH to afford compound **5** and can also be activated by isobutyl chloroformate and *N*-methylmorpholine and then coupled with NH_2OH to yield its hydroxamate acid derivatives.

In addition, as shown in Scheme 2, the methyl group of 3,4,5-trimethoxybenzoic acid part was deprotected to free the phenyl group with BBr_3 as reagent to study the interaction between free gallic acid with the active site of enzyme.

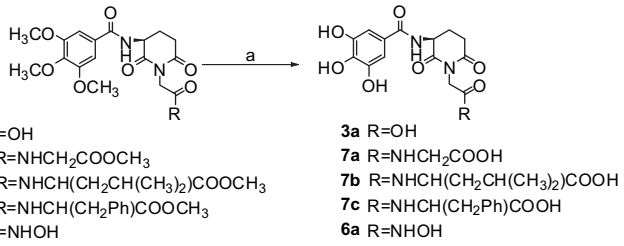
3. Result and discussion

The preliminary pharmacological studies of all the target compounds have been investigated on enzymatic inhibition of APN and the results are listed in Table 1. Compared with those compounds in the previous work, preliminary result showed that most of target compounds displayed improved inhibitory activity than those reported before [12].

As shown in the results, the activity of piperidinedione inhibitors toward APN was strongly dependent on the nature of the moiety present at R_2. Among simpler carboxylate analogues **3**, **5** and **6** (R_2 = OH, OMe or NHOH, respectively), hydroxamic acids **6**



Scheme 1. a. Ac_2O, 55–60 °C, 60%; b. Glycine, DMF, Microwave, 110 °C, 1 h, 57%; c. Methyl ester of amino acids, EDCI, HOBt, DCM/DMSO, 56–85%; d. $SOCl_2$, CH_3OH, 98%; e. Et_3N, isobutyl chloroformate, NH_2OH·HCl, 60%.



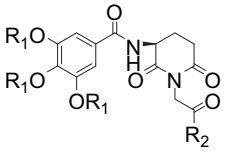
3 R=OH
4a R=NHCH$_2$COOCH$_3$
4b R=NHCH(CH$_2$CH(CH$_3$)$_2$)COOCH$_3$
4c R=NHCH(CH$_2$Ph)COOCH$_3$
6 R=NHOH

3a R=OH
7a R=NHCH$_2$COOH
7b R=NHCH(CH$_2$CH(CH$_3$)$_2$)COOH
7c R=NHCH(CH$_2$Ph)COOH
6a R=NHOH

Scheme 2. a. (i) BBr_3, DCM, −78 °C; (ii) H_2O.

and **6a** appeared the most tightly bound with the IC_{50} values an order of magnitude lower than the ones calculated for counterpart acid **3** and ester **5** (3.1 μM and 4.3 μM vs. 50–60 μM). The derivatives extended with an additional amino ester residue showed further interesting structure–activity relationships. The presence of an aliphatic side chain portion was not yet discriminating for the efficiency of studied compounds. The analogues of Val, Ala, Leu and Ile (**4a–f**) displayed similar to each other, moderate potency (IC_{50} in the range of 20–50 μM), only slightly improved in comparison to non-substituted Gly **4a** (IC_{50} = 55.4 μM). Introduction of residues containing heteroatoms (for example: Arg, Asp, Lys, Cys, etc.) did not change the situation. However, arylalkyl Phe analogue exhibited enhanced activity, with 5-fold preference for natural L isomer (IC_{50} = 11.0 μM vs. 46.2 μM for the D form). Finally, the most

Table 1
In vitro enzyme assay results for compounds **3–7** and bestatin against APN.



No	Substituents		IC_{50}/μM[a]
	R_1	R_2	APN
3	CH_3	OH	49.8 ± 5.5
3a	H	OH	47.3 ± 6.7
4a	CH_3	Gly–OCH_3	55.4 ± 3.5
4b	CH_3	Val–OCH_3	21.4 ± 2.6
4c	CH_3	Leu–OCH_3	46.2 ± 3.2
4d	CH_3	Ile–OCH_3	49.3 ± 2.9
4e	CH_3	β-Ala–OCH_3	53.7 ± 8.4
4f	CH_3	Ala–OCH_3	18.9 ± 4.4
4g	CH_3	Arg (NO_2)–OCH_3	42.0 ± 7.6
4h	CH_3	Met–OCH_3	20.7 ± 2.7
4i	CH_3	Lys (Z)–OCH_3	38.1 ± 3.8
4j	CH_3	Tyr–OCH_3	44.8 ± 5.2
4k	CH_3	Trp–OCH_3	43.1 ± 3.2
4l	CH_3	His–OCH_3	5.2 ± 2.1
4m	CH_3	D-Phe–OCH_3	46.2 ± 6.4
4n	CH_3	Thr–OCH_3	51.9 ± 3.9
4o	CH_3	L-Phe–OCH_3	11.0 ± 3.0
4p	CH_3	Cys–OCH_3	50.3 ± 4.3
4q	CH_3	2-Cl-Ala–OCH_3	50.0 ± 4.2
4r	CH_3	Asp–(OCH_3)$_2$	21.7 ± 2.1
5	CH_3	–OCH_3	63.4 ± 3.2
6	CH_3	–NHOH	3.1 ± 0.7
6a	H	–NHOH	4.3 ± 0.4
7a	H	Gly–OH	63.2 ± 1.3
7b	H	Leu–OH	55.4 ± 2.7
7c	H	L-Phe–OH	10.3 ± 1.4
Bestatin			2.4 ± 0.5

[a] Values are means of three experiments, standard deviation is given.

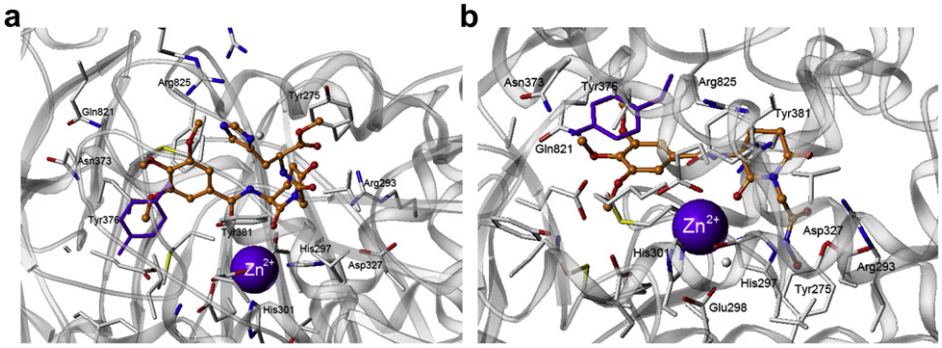


Fig. 2. Docking modes for **4l** (a) and **6** (b) with APN (PDB code: 2DQM).

favorable appeared heteroaromatic system of histidine derived compound **4l**. The latter one ($IC_{50} = 5.2\ \mu M$) together with hydroxamate **6** displayed similar efficiency to bestatin ($IC_{50} = 2.4\ \mu M$). Interestingly, neither deprotection of the methoxy groups in the gallic acid part, nor their simultaneous removal together with this at the C-termini, caused any significant change in activity in comparison to blocked counterparts (compare **3** vs. **3a**, **6** vs. **6a**, **4a** vs. **7a**, etc.).

In order to obtain further insight into the interaction of target compound with APN, the most active compounds **4l** and **6** were built and docked into the active site of APN (PDB code: 2DQM) using Gold4.0. The binding studies showed that the aromatic ring of gallic acid for part A is favorable as it can form a π–π interaction with the Tyr376 located in the active site, which is the same to that of bestatin, Fig. 2. The oxygen atoms from trimethoxyl group interact with Arg825, Gln821 and Asn373. Two carbonyl groups (C═O from C-2 on the cyclic-imide ring and gallic acid) bind to the zinc ion and form a five-membered ring in the active pocket with distance of 2.45 Å and 2.04 Å, respectively. In addition, the C-2 carbonyl group also interacts with the hydroxyl group of Tyr381 by hydrogen bond which would be benefit to stabilize of the reaction intermediate with the zinc ion. As for part B, Tyr275, Arg293 and Asp327 in the active site form hydrogen bond with either **4l** (the methyl ester part, Fig. 2a) or **6** (hydroxamate acid group, Fig. 2b). Additionally, NHOH of **6** can form hydrogen bond interaction with His297 (2.03 Å) that is the essential amino acids of the conserved sequence (HEXXHX18E) in the catalytic domain that is well conserved in peptidase M1 family.

To investigate the biological function of target compounds, cells cytostatic activity assay was carried out in human leukemia HL-60 cell lines, which highly express APN, using MTT assay (see Experimental section). The concentrations inducing a 50% inhibition of cell growth (IC_{50}) in μM are reported. The data indicated that several compounds have the cytostatic activity, among which **4g** and **6** displayed potent inhibitory activity, Fig. 3, which are more effective than the positive control (the IC_{50} for bestatin was 245 μM). Although the purpose of our research is to design and synthesize enzyme inhibitors, interestingly there were still several other compounds induce the growth of HL-60 cells (results will be published later).

To evaluate the ability of target compounds to inhibit the metastasis of cancer cells in vivo, anti-metastasis assay of H22 hepatic cells was carried out. The results are shown in Fig. 4. Compound **6** displayed higher inhibitory activity ($P < 0.001$) than that of bestatin with the inhibitory rate of 81.56% and 60.25%, respectively, which means that it is of great value for further preclinical research.

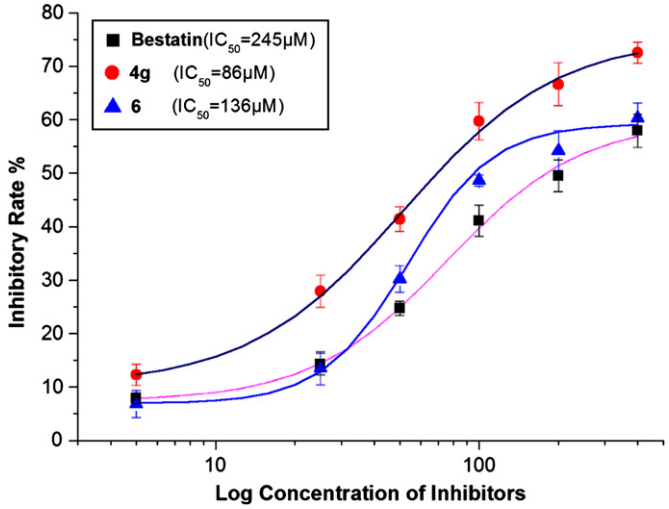


Fig. 3. In vitro cytostatic activity of the compound in HL-60 cells.

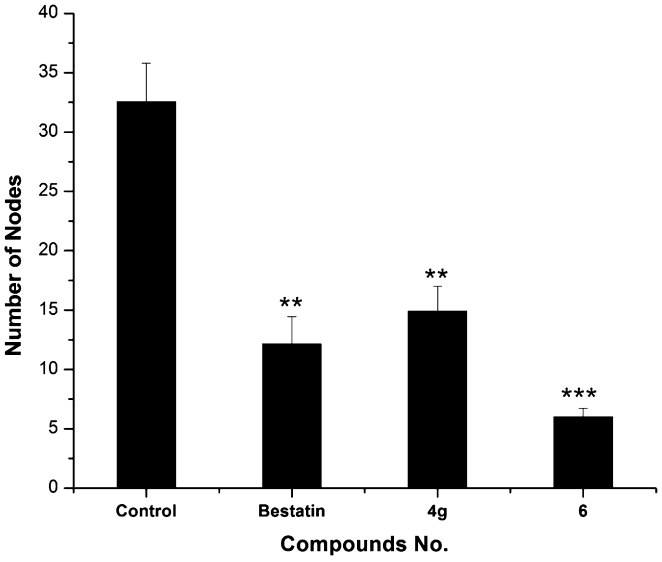


Fig. 4. In vivo anti-metastasis assay of H22 hepatic cells. (The difference for the number of metastasized nodes on lung surface between drug groups and negative control group is analyzed using one-way ANOVA method. $^{**}P < 0.01$ or $^{***}P < 0.001$ indicates significant differences from control group.)

4. Conclusion

In summary, our studies have shown that cyclic-imide is a novel scaffold for the design of aminopeptidase N inhibitors. Most of the compounds possess potent APN inhibitory activity and the most potent compounds, **4l** and **6**, exhibited good enzymatic inhibition against APN. With the combination of the requirement of the active site of APN, this interesting activity profile may also guide the design of new, specific inhibitors of aminopeptidases with 'one-zinc' active site.

5. Experimental

5.1. Chemistry

Unless otherwise stated, materials were obtained from commercial suppliers and purified according to the methods of chemical reagents. Melting points were determined on Boetius apparatus and were not corrected. Column chromatography was carried out on silica gel (200–300 mesh). TLCs were performed on precoated silica gel plates, and the resulting chromatograms were visualized under UV light at 254 nm. Proton NMR spectra were recorded on a Bruker DRX spectrometer operating at 400 MHz. Measurements were made in $CDCl_3$ or DMSO-d_6 solutions. Proton chemical shifts are reported in relation to tetramethylsilane (TMS) used as internal standard with the unit of δ in ppm and J in Hertz. ESI-MS were measured on an API 4000 spectrometer.

5.1.1. (S)-2-(3,4,5-Trimethoxybenzamido)pentanedioic acid (**1**) [12]

The key intermediate *N*-(3,4,5-trimethoxybenzoyl)-glutamic acid anhydride (**2**) was prepared starting from 3,4,5-trihydroxybenzoic acid via a three-step reaction according to the literature.

19.7 g (0.105 mol) of glutamic acid and 19.2 g (0.181 mol) of sodium carbonate were dissolved in 150 ml water. The mixture is cooled to 0–2 °C on ice–water bath and to the mechanically stirred solution was added dropwise 0.1 mol 3,4,5-trimethoxybenzoyl chloride in 150 ml anhydrous benzene over 2 h and kept stirring for about 5 h. The aqueous phase was acidified by concentrated hydrochloride to pH 4 and extracted by 100 ml of chloroform. The aqueous phase was acidified further and the product precipitated in a great quantity. Filter and wash to neutrality with ice–water. The crude product was recrystallized in EtOAc to obtain 23.7 g (yield 69.5%) white crystal.

5.1.2. (S)-N-(2,6-Dioxo-tetrahydro-2H-pyran-3-yl)-3,4,5-trimethoxybenzamide (**2**)

Compound **1** (11.4 g, 33.4 mmol) was suspended in 80 ml Ac_2O. After stirring for 6 h at 55–60 °C, the resulting mixture was filtered, and to the filtrate was added equal volume of ethyl ether:petroleum ether (2:1) and cool in fridge overnight. 5.6 g (yield 52%) white crystal solid was obtained by filtrating.

5.1.3. (S)-2-(2,6-Dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetic acid (**3**)

8.7 g (27 mmol) of compound **2** and 2.03 g (27 mmol) of glycine were suspended in 70 ml DMF. The mixture was heated by microwave at 1000 W, 110 °C for about 3 h. Cool the mixture to room temperature and equal volume of water was added, the solution was acidified to pH 3 and cooled in fridge overnight. 5.7 g (yield 55.7%) of white solid were obtained. mp 246.5 °C (decomp). ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.34 (s, 1H), 8.88 (d, 1H, J = 8.3 Hz), 7.17 (s, 2H), 4.53 (m, 1H), 4.42 (s, 2H), 3.83 (s, 6H), 3.71 (s, 3H), 2.18 (m, 2H), 2.11 (m, 2H).

5.1.4. General procedure for the preparation of **4a**–**r**

To a mixture of **3** and 1-hydroxy-1*H*-benzotriazole (HOBt) (1.5 equiv) in DCM (20 ml) and DMSO (3 ml) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) (1.5 equiv) at 0 °C. To the clear solution were added amino acid derivatives and Et_3N (3.5 equiv) and stirred for 10–15 h at 30 °C. The mixture was monitored by TLC. The reaction mixture was diluted with DCM, washed successively with 0.5% Na_2CO_3, 1 N HCl and brine, and dried over anhydrous Na_2SO_4. Filtration and concentration in vacuum gave **4** as a crude solid and then purified by column chromatography.

5.1.4.1. (S)-Methyl 2-(2-(2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl)acetamido)acetate (***4a***).

The compound was prepared from **3** (1.9 g, 5.0 mmol) and (*S*)-methyl 2-aminoacetate hydrochloride (0.75 g, 6.0 mmol) according to the general procedure to yield **4a** as white solid (1.41 g, 62.7%): mp 95.7–100.6 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.05 (s, 2H), 4.84 (m, 1H), 4.37 (dd, 2H, J = 25.5, 15.6), 4.06 (s, 2H,), 3.91 (s, 6H), 3.89 (s, 3H), 3.77 (s, 3H), 2.93 (m, 2H), 2.63 (m, 1H), 2.07 (m, 1H); ESI-MS m/z $[M+H]^+$ 452.5.

5.1.4.2. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)-piperidin-1-yl)acetamido)-3-methylbutanoate (***4b***).

The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-3-methylbutanoate hydrochloride (0.40 g, 2.38 mmol) according to the general procedure to yield **4b** as white solid (0.75 g, 79.8%): mp 110.5–112.0 °C, ^{1}H NMR: (400 MHz, DMSO-d_6): δ 8.81 (d, 1H, J = 8.3 Hz), 8.22 (d, 1H, J = 8.0 Hz,), 7.210 (s, 2H), 4.93 (m, 1H), 4.34 (s, 2H), 4.16 (s, 1H), 3.83 (s, 6H), 3.71 (s, 3H), 3.67 (s, 3H), 3.30 (s, 3H), 3.09 (m, 1H), 2.75 (m, 2H), 2.03 (m, 2H), 0.86 (d, 6H); ESI-MS m/z $[M+H]^+$ 494.5.

5.1.4.3. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)-piperidin-1-yl)acetamido)-4-methylpentanoate (***4c***).

The compound was prepared from **3** (1.9 g, 5.0 mmol) and (*S*)-methyl 2-amino-4-methylpentanoate hydrochloride (1.01 g, 5.6 mmol) according to the general procedure to yield **4c** as white solid (1.72 g, 67.8%): mp 85.3–87.4 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.82 (d, 1H), 8.40 (d, 1H), 7.22 (s, 2H), 4.91 (m, 1H), 4.30 (m, 3H), 3.83 (s, 6H), 3.71 (s, 3H), 3.61 (s, 3H), 3.09 (m, 1H), 3.03 (m, 1H), 2.14 (m, 1H), 2.03 (m, 1H), 1.86 (m, 1H), 1.60 (m, 2H), 0.85 (dd, 6H); ESI-MS m/z $[M+H]^+$ 508.5.

5.1.4.4. (2S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)-3-methylpentanoate (***4d***).

The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-3-methylpentanoate hydrochloride (0.73 g, 4.0 mmol) according to the general procedure to yield **4d** as white solid (0.75 g, 79.8%): mp 84.0–85.7 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.05 (s, 2H), 4.81 (m, 1H), 4.56 (s, 3H), 3.91 (s, 6H), 3.89 (s, 3H), 3.74 (s, 3H), 2.88 (m, 3H), 2.64 (m, 1H), 2.04 (m, 1H), 1.90 (m, 1H), 1.43 (m, 1H), 1.23 (m, 1H), 0.91 (m, 6H); ESI-MS m/z $[M+H]^+$ 508.5.

5.1.4.5. (S)-Methyl 3-(2-(2,6-dioxo-3-(3,4,5-trimethoxybenzamido)-piperidin-1-yl)acetamido)propanoate (***4e***).

The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 3-aminopropanoate hydrochloride (0.56 g, 4.0 mmol) according to the general procedure to yield **4e** as white solid (0.80 g, 87.6%): mp 139.3–140.5 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.82 (d, 1H, J = 8.3 Hz), 8.16 (d, 1H, J = 8.0 Hz), 7.22 (s, 2H), 4.93 (m, 1H), 4.23 (s, 2H), 3.84 (s, 6H), 3.72 (s, 3H), 3.60 (s, 3H), 3.29 (m, 2H), 2.96 (m, 1H), 2.75 (m, 1H), 2.45 (t, 2H), 2.24 (m, 1H), 2.03 (m, 1H); ESI-MS m/z $[M+H]^+$ 466.6.

5.1.4.6. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)-piperidin-1-yl)acetamido)propanoate (***4f***).

The compound was prepared from **3** (1.9 g, 5.0 mmol) and (*S*)-methyl 2-aminopropanoate hydrochloride (0.84 g, 6 mmol) according to the general procedure to yield **4f** as white solid (1.65 g, 70.9%): mp 98.8–101.6 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.84 (d, 1H), 8.49 (d, 1H), 7.22 (s, 2H), 4.93 (m,

1H), 4.29 (m, 3H), 3.83 (s, 6H), 3.71 (s, 3H), 3.60 (s, 3H), 2.95 (m, 1H), 2.75 (m, 1H), 2.17 (m, 1H), 1.98 (m, 1H), 1.27 (d, 3H); ESI-MS *m/z* $[M+H]^+$ 466.4.

*5.1.4.7. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)-piperidin-1-yl)acetamido)-5-(3-nitroguanidino)pentanoate (**4g**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-5-(3-nitroguanidino)pentanoate hydrochloride (0.81 g, 3.0 mmol) according to the general procedure to yield **4g** as white solid (0.79 g, 66.3%): mp 53.9–55.1 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.82 (d, 1H, $J = 8.3$ Hz), 8.47 (d, 1H, $J = 8.0$ Hz), 7.83 (d, 1H), 7.22 (s, 2H), 4.92 (m, 1H), 4.32 (s, 2H), 4.27 (s, 1H,), 3.83 (s, 6H), 3.71 (s, 3H), 3.69 (s, 3H), 3.14 (m, 2H), 2.93 (m, 1H), 2.75 (m, 1H), 2.186 (m, 1H), 2.03 (m, 1H), 1.62 (m, 4H); ESI-MS *m/z* $[M+H]^+$596.6.

*5.1.4.8. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)-piperidin-1-yl)acetamido)-4-(methylthio)butanoate (**4h**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-4-(methylthio)butanoate hydrochloride (0.80 g, 4.0 mmol) according to the general procedure to yield **4h** as white solid (0.67 g, 63.7%): mp 91.4–93.8 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.82 (d, 1H, $J = 8.3$ Hz), 8.15 (d, 1H, $J = 8.0$ Hz), 7.23 (s, 2H), 4.93 (m, 1H), 4.39 (m, 1H), 4.31 (s, 2H), 3.83 (s, 6H), 3.71 (s, 3H), 3.63 (s, 3H), 2.95 (m, 1H), 2.78 (m, 1H), 2.46 (m, 2H), 2.11 (m, 1H), 2.02 (s, 3H), 1.93 (m, 3H); ESI-MS *m/z* $[M+H]^+$526.6.

*5.1.4.9. (S)-Methyl 6-(benzyloxycarbonylamino)-2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)hexanoate (**4i**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-6-(benzyloxycarbonylamino)hexanoate hydrochloride (0.80 g, 4.0 mmol) according to the general procedure to yield **4i** as white solid (0.89 g, 67.8%): mp 79.8–82.3 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.81 (d, 1H), 8.15 (d, 1H, $J = 8.0$ Hz), 7.29 (m, 5H), 7.22 (s, 2H), 5.05 (s, 2H), 4.99 (s, 2H), 4.95 (s, 1H), 4.31 (dd, 2H,), 4.21 (m, 1H), 3.83 (s, 6H), 3.71 (s, 3H), 3.61 (s, 3H), 2.98 (m, 3H), 2.79 (m, 1H), 2.15 (m, 1H), 2.03 (m, 1H), 1.81 (m, 2H), 1.40–1.36 (m, 2H), 1.27 (m, 2H); ESI-MS *m/z* $[M+H]^+$657.6.

*5.1.4.10. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)-3-(4-hydroxyphenyl)propanoate (**4j**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-3-(4-hydroxyphenyl)propanoate hydrochloride (0.92 g, 4.0 mmol) according to the general procedure to yield **4j** as white solid (0.72 g, 64.6%): mp 95.6–97.8 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.20 (s, 1H), 8.81 (d, 1H, $J = 8.3$ Hz), 8.41 (d, 1H, $J = 8.0$ Hz), 7.22 (s, 2H), 6.96 (d, 2H), 6.61 (d, 2H), 4.96 (m, 1H), 4.36 (m, 1H), 4.28 (s, 2H), 3.83 (s, 6H), 3.71 (s, 3H), 3.56 (s, 3H), 3.23 (m, 1H), 2.67 (m, 5H), 2.02 (m, 1H); ESI-MS *m/z* $[M+H]^+$558.6.

*5.1.4.11. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)-3-(1H-indol-3-yl)propanoate (**4k**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-3-(1*H*-indol-3-yl)propanoate hydrochloride (0.76 g, 3.0 mmol) according to the general procedure to yield **4k** as white solid (0.67 g, 57.7%): mp 91.3–93.2 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.87 (s, 1H), 8.83 (t, 1H, $J = 7.6$ Hz), 8.51 (d, 1H, $J = 7.5$ Hz), 7.47 (d, 1H, $J = 7.8$ Hz), 7.32 (d, 1H, $J = 7.9$ Hz), 7.23 (s, 2H), 7.13 (s, 1H), 7.05 (t, 1H, $J = 7.1$ Hz), 6.98 (t, 1H, $J = 7.9$ Hz), 4.98 (m, 1H), 4.52 (d, 1H, $J = 6.56$ Hz), 4.31 (s, 2H,), 3.83 (s, 6H), 3.71 (s, 3H), 3.56 (s, 3H), 3.12 (m, 3H), 2.77 (m, 1H), 2.18 (m, 1H), 2.02 (m, 1H); ESI-MS *m/z* $[M+H]^+$581.5.

*5.1.4.12. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)-3-(1H-imidazol-5-yl)propanoate (**4l**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-3-(1*H*-imidazol-4-yl)propanoate hydrochloride (0.97 g, 4.0 mmol) according to the general procedure to yield **4l** as white solid (0.70 g, 65.9%): mp 111.7–113.5 °C, ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.4 (d, 1H), 8.84 (d, 1H, $J = 8.3$ Hz), 8.73 (d, 1H), 8.32 (d, 1H, $J = 8.0$ Hz), 7.66 (s, 1H), 7.23 (s, 2H), 4.96 (m, 1H), 4.52 (m, 1H), 4.34 (s, 2H), 3.83 (s, 6H), 3.71 (s, 3H), 3.63 (s, 3H), 3.20 (m, 3H), 2.76 (m, 1H), 2.14 (m, 1H), 2.03 (m, 1H); ESI-MS *m/z* $[M+H]^+$532.6.

*5.1.4.13. (R)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)-3-phenylpropanoate (**4m**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*R*)-methyl 2-amino-3-phenylpropanoate hydrochloride (0.86 g, 3.0 mmol) according to the general procedure to yield **4m** as white solid (0.74 g, 68.3%): mp 69.3–71.2 °C, ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25 (m, 7H), 4.82 (m, 2H), 4.48 (dd, 2H) 3.90 (s, 6H), 3.87 (s, 3H), 3.73 (s, 3H), 3.14 (d, 2H), 2.94 (m, 2H), 2.62 (m, 1H), 2.03 (m, 1H); ESI-MS *m/z* $[M+H]^+$542.6.

*5.1.4.14. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)-3-hydroxypropanoate (**4n**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-3-hydroxypropanoate hydrochloride (0.62 g, 4.0 mmol) according to the general procedure to yield **4n** as white solid (0.59 g, 61.3%): mp 158.9–160.1 °C, ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.82 (d, 1H, $J = 8.3$ Hz), 8.43 (d, 1H, $J = 8.0$ Hz), 7.23 (s, 2H), 5.08 (m, 1H), 4.92 (m, 1H), 4.35 (m, 2H), 3.83 (s, 6H), 3.71 (s, 3H), 3.67 (m, 1H), 3.63 (s, 3H), 3.60 (m, 1H), 2.96 (m, 1H), 2.78 (m, 1H), 2.17 (m, 1H), 2.03 (m, 1H); ESI-MS *m/z* $[M+H]^+$482.6.

*5.1.4.15. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)-3-phenylpropanoate (**4o**).* The compound was prepared from **3** (1.9 g, 5.0 mmol) and (*S*)-methyl 2-amino-3-phenylpropanoate hydrochloride (1.30 g, 6 mmol) according to the general procedure to yield **4o** as white solid (2.6 g, 96.0%): mp 68.5–70.6 °C, ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25 (m, 7H), 4.81 (m, 2H), 4.48 (dd, 2H) 3.90 (s, 6H), 3.87 (s, 3H), 3.73 (s, 3H), 3.14 (d, 2H), 2.95 (m, 2H), 2.63 (m, 1H), 2.03 (m, 1H); ESI-MS *m/z* $[M+H]^+$542.6.

*5.1.4.16. (S)-Methyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)-3-mercaptopropanoate (**4p**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-3-mercaptopropanoate hydrochloride (0.69 g, 4 mmol) according to the general procedure to yield **4p** as white solid (0.53 g, 53.3%): mp 88.5–90.0 °C, ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.83 (d, 1H, $J = 8.0$ Hz), 8.16 (d, 1H, $J = 8.4$ Hz), 7.23 (s, 2H), 4.93 (m, 1H), 4.49 (m, 1H), 4.33 (s, 2H,), 3.83 (s, 6H), 3.71 (s, 3H), 3.67 (s, 3H), 3.08 (m, 1H), 2.97 (m, 2H), 2.76 (m, 1H), 2.20 (m, 1H), 2.02 (m, 1H); ESI-MS *m/z* $[M+H]^+$498.5.

*5.1.4.17. (S)-Methyl 3-chloro-2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)propanoate (**4q**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-methyl 2-amino-3-chloropropanoate hydrochloride (0.52 g, 3.0 mmol) according to the general procedure to yield **4u** as white solid (0.68 g, 68.0%): mp 116.5–117.4 °C, ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.81 (d, 1H, $J = 8.1$ Hz), 8.15 (d, 1H, $J = 8.4$ Hz), 7.23 (s, 2H), 4.94 (s, 1H), 4.49 (m, 2H), 4.12 (m, 1H), 3.87 (m, 1H), 3.77 (s, 6H), 3.71 (s, 3H), 3.67 (s, 3H), 3.30 (s, 2H), 2.96 (m, 1H), 2.77 (m, 1H), 2.21 (m, 1H), 2.031 (m, 1H); ESI-MS *m/z* $[M+H]^+$499.5, $[M+2]^+$501.5.

*5.1.4.18. (S)-Dimethyl 2-(2-((S)-2,6-dioxo-3-(3,4,5-trimethoxybenzamido)piperidin-1-yl)acetamido)succinate (**4r**).* The compound was prepared from **3** (0.76 g, 2.0 mmol) and (*S*)-dimethyl 2-aminosuccinate hydrochloride (0.59 g, 3.0 mmol) according to

the general procedure to yield **4r** as white solid (0.76 g, 72.6%): mp 88.8–90.7 °C, ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.83 (d, 1H, $J = 8.32$ Hz), 8.16 (d, 1H, $J = 8.44$ Hz), 7.23 (s, 2H), 4.921 (m, 1H), 4.63 (m, 1H, $J = 6.74$ Hz), 4.3 (dd, 2H), 3.83 (s, 6H), 3.71 (s, 3H), 3.61 (s, 6H), 2.96 (m, 1H), 2.74 (m, 3H), 2.18 (m, 1H), 2.01 (m, 1H); ESI-MS m/z $[M + H]^+$524.6.

*5.1.5. (S)-Methyl 2-(2,6-dioxo-3-(3,4,5-trimethoxybenzamido) piperidin-1-yl)acetate (**5**)*

Compound **3** (3.8 g, 10 mmol) was suspended in 100 ml MeOH. To the mixture was added $SOCl_2$ (1.5 ml) dropwise. During the addition, the inner temperature was controlled under 3 °C by ice–water bath. After 1 h, the reaction mixture was stirred at room temperature for 10 h and condensed by rotary evaporation to obtain white solid 3.76 g (yield 95.4%): mp 135.1–137.0 °C, ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.81 (d, 1H), 8.32 (d, 1H), 7.23 (s, 2H), 4.95 (m, 1H,), 4.55 (dd, 2H), 3.83 (s, 6H), 3.71 (s, 3H), 3.65 (s, 3H), 3.02 (m, 1H), 2.80 (m, 1H), 2.20 (m, 1H), 2.04 (m, 1H); ESI-MS m/z $[M + H]^+$ 395.4.

*5.1.6. (S)-N-(1-(2-(Hydroxyamino)-2-oxoethyl)-2,6-dioxpiperidin-3-yl)-3,4,5-trimethoxybenzamide (**6**)*

0.2 g of NH_2OH·HCl was dissolved in 1.5 ml anhydrous MeOH and to the clear solution was added 0.5 ml of Et_3N. The carboxyl acid derivative **3** (2.0 mmol) was dissolved in 20 ml anhydrous THF and to control the inner temperature not higher than −20 °C. 2.0 equiv of Et_3N was added slowly, and after 5 min, 1.01 equiv of isobutyl chloroformate was added. The mixture was stirred at −20 °C for another 10 min and to the mixture was added slowly MeOH solution with NH_2OH after which the mixture was stirred at −20 °C for 15 min and then kept at 0 °C for 2 h. The solid formed was filtered by 2.0 g celite and the filtrate was condensed and dissolved in 30 ml of AcOEt. The organic phase was then washed in turn by 0.05%$NaHCO_3$ (10 ml × 2), 5% Citric acid (10 ml × 1), brine and then dried over Na_2SO_4. Purified by column chromatography to obtain **6** 0.37 g (47.5%) as white crystal: mp 182.8–185.3 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.31 (s, 1H), 8.44 (d, 1H, $J = 7.9$ Hz), 8.23 (d, 1H, $J = 5.7$ Hz,), 7.24 (s, 2H), 4.50 (m, 1H), 3.84 (s, 6H), 3.75 (s, 2H), 2.34 (m, 2H), 2.07 (m, 1H), 1.93 (m, 1H); ESI-MS m/z $[M + H]^+$ 396.2.

*5.1.7. General procedure for the deprotection of methyl group of 3,4,5-trimethoxybenzamide compounds (**3**, **4a–c**, **6**)*

The round-bottom flask was charged with 1.0 mmol of trimethoxybenzamide compounds under N_2. 10 ml of anhydrous DCM was added and cooled at dry-ice acetone bath to −78 °C and stirred for 15 min. To the mixture was added dropwise 3.1 mmol of BBr3 in anhydrous DCM, and kept stirring at −78 °C for another 10 min and then 4 h at room temperature. The mixture was quenched by the addition of saturated $NaHSO_3$ solution and extracted with DCM. The crude products were recrystallized by EtOH.

*5.1.7.1. (S)-2-(2,6-Dioxo-3-(3,4,5-trihydroxybenzamido)piperidin-1-yl)acetic acid (**3a**).* Yellow solid 0.18 g, yield 53.1%: mp 187.2–189.4 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.31 (s, 1H), 9.01 (s, 3H), 8.83 (d, 1H), 6.81 (s, 2H), 4.69 (m, 1H), 4.27 (m, 2H), 2.77 (m, 1H), 2.53 (m, 1H), 2.07 (m, 1H), 1.96 (m, 1H); ESI-MS m/z $[M + H]^+$ 339.3.

*5.1.7.2. (S)-2-(2-(2,6-Dioxo-3-(3,4,5-trihydroxybenzamido)piperidin-1-yl)acetamido)acetic acid (**7a**).* Off-white solid 0.20 g, yield 50.6%: mp 112.8–115.2 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.61 (d, 1H), 8.20 (d, 1H), 8.03 (s, 3H), 6.85 (s, 2H), 4.92 (m, 1H), 4.27 (m, 3H), 2.31 (m, 2H), 1.97 (m, 2H); ESI-MS m/z $[M + H]$ 396.4.

*5.1.7.3. (S)-2-(2-((S)-2,6-Dioxo-3-(3,4,5-trihydroxybenzamido)piperidin-1-yl)acetamido)-4-methylpentanoic acid (**7b**).* Yellow powder 0.29 g, yield 64.4%; mp 127.3–129.4 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.64 (d, 1H), 8.22 (d, 1H), 8.04 (s, 3H), 6.86 (s, 2H), 4.91 (m, 1H), 4.25 (m, 3H), 2.31 (m, 2H), 1.97 (m, 2H), 1.58 (m, 3H), 0.86 (dd, 6H); ESI-MS m/z $[M + H]^+$ 452.5.

*5.1.7.4. (S)-2-(2-((S)-2,6-Dioxo-3-(3,4,5-trihydroxybenzamido) piperidin-1-yl)acetamido)-3-phenylpropanoic acid (**7c**).* Yellow solid 0.21 g, yield 43.3%: mp 102.5–104.9 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.50 (m, 1H), 8.38 (s, 1H), 7.24 (m, 5H), 6.85 (s, 2H), 4.82 (m, 1H), 4.38 (m, 2H), 4.25 (s, 2H), 3.00 (m, 2H), 1.98 (m, 2H); ESI-MS m/z $[M + H]^+$486.6.

*5.1.7.5. (S)-3,4,5-Trihydroxy-N-(1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)benzamide (**6a**).* White solid 0.20 g yield 57.1%: mp 210 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.78 (s, 1H), 9.01 (s, 3H), 8.83 (d, 1H), 8.06 (d, 1H), 6.85 (s, 2H), 4.67 (m, 1H), 4.33 (m, 2H), 2.77 (m, 1H), 2.53 (m, 1H), 2.07 (m, 1H), 1.96 (m, 1H); ESI-MS m/z $[M + H]^+$: 354.3.

5.2. Biological materials and methods

5.2.1. Cell line and cell culture

The human leukemia cell line, HL-60 was obtained from the Institute of National Cancer Research of China (Beijing, China). Cells were maintained in RPMI-1640 supplemented with 10%(v/v) heat–inactivated fetal bovine serum, penicillin–streptomycin (100 IU/ml–100 μg/ml), 2 mM glutamine, and 10 mM Hepes buffer at 37 °C in a humid atmosphere (5% CO_2–95% air). Cell viability was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma, USA) assay as described elsewhere.

5.2.2. In vitro APN enzyme assay [19]

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 pre-incubated 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.

5.2.3. In vitro cytostatic activity [20]

The inhibitors were dissolved in culture medium and diluted to the required concentration, and the in vitro cytostatic activity was evaluated by MTT assay. Briefly, the human leukemia cell line HL-60 was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and incubated at 37 °C in a CO_2 incubator. Cells were seeded on a 96-well plate (10^4 cells per well). After 4 h incubation, Cyclic-imide peptidomimetic inhibitors were then added to the wells to achieve final concentration of 800, 600, 400, 200 and 100 μg/ml Control wells were prepared by addition of culture medium. At same time, the bestatin (Apeloa Pharma/Kangyu Pharma, China) was used as positive control. The plates were incubated for 48 h. Upon completion of the incubation, 1% of 0.5 mg/ml MTT was added to each well and incubated for an additional 4 h. After centrifugalization, medium was removed and 100 μl DMSO was added. Absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay reader (Model 680, BIO-RAD), and absorbance at 630 nm was used as a reference. The percent growth inhibitory rate of treated cells was calculated by $(OD_{drug-free\ control} - OD_{tested})/OD_{drug-free\ control} \times 100\%$, where OD is

the mean value calculated using the data from three replicate tests. The IC_{50} values were determined by plotting the percentage viability vs. concentration on a logarithmic graph and reading off the concentration at which 50% of cells viable relative to the control. Each experiment was repeated at least three times to get the mean values. The curves were defined using Origin 7.5 software.

5.2.4. Anti-metastasis assay in vivo [21]

Mice bearing H22 tumor were injected via the caudal vein and randomly divided into 4 groups according to the method reported previously. The animals of the control group were treated with the same volume of CMC-Na, while the other groups were given the inhibitors (**4g**, **6** and bestatin) by oral administration, at a dose of 50 mg/kg/day, 6 days/week for 2 weeks. The mice were then weighed and sacrificed for autopsy immediately. The lungs with tumor nodes were removed, weighed, and then placed in Bouin stationary solution (saturated 2,4,6-trinitrophenol solution/formaldehyde/glacial acetic acid = 15:5:1). One day later, the metastasized nodes on the surface of lungs were counted.

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