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RESEARCH ARTICLE

Design, synthesis and biological evaluation of novel *L*-isoserine tripeptide derivatives as aminopeptidase N inhibitors

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Abstract

Aminopeptidase N (APN/CD13) is one of the essential proteins for tumour invasion, angiogenesis and metastasis as it is over-expressed on the surface of different tumour cells. Based on our previous work that *L*-isoserine dipeptide derivatives were potent APN inhibitors, we designed and synthesized *L*-isoserine tripeptide derivatives as APN inhibitors. Among these compounds, one compound **16l** ($IC_{50} = 2.51 \pm 0.2$ μM) showed similar inhibitory effect compared with control compound **Bestatin** ($IC_{50} = 6.25 \pm 0.4$ μM) and it could be used as novel lead compound for the APN inhibitors development as anticancer agents in the future.

Keywords: Aminopeptidase N, inhibitors, *L*-isoserine tripeptide derivatives, anticancer agents, synthesis

Introduction

Aminopeptidase N (APN; EC 3.4.11.2) is a zinc dependent type II membrane-bond exopeptidase[1,2], which is selectively expressed on the surface of cells such as myeloid progenitors and monocytes, epithelial cells of the intestine and kidney, synaptic membranes in the central nervous system, fibroblasts, endothelial cells, epithelial cells[3–6] and so on. Aminopeptidase N which is expressed on tumour cells can degrade the extra cellular matrix (ECM) and considered as the first step of tumour proliferation and matastasis[7]. Therefore, APN is a promising target for cancer therapy and APN inhibitors may be of great clinical significance to suppress the invasion, angiogenesis and metastasis of tumour cells.

Up to now, a quantity of APN inhibitors such as Bestatin, Probestin[8], Amatatin[9], Prebestin[8], Lapstatin[10], AHPA-Val[11] have been reported. The first APN inhibitor **Bestatin** was isolated from a culture filtrate of *Streptomyces olivoreticuli* in 1976[12], and now generally used as the positive control in the assay of small molecule APN inhibitors. The co-crystal complex of **Bestatin** (AHPA-Leu) and APN from *Escherichia coli* was reported in 2006, from which we can know that the hydroxyl and carbonyl belong to zinc binding group (ZBG), the phenyl ring in AHPA fragment can insert into the S1 pocket and the amino group in AHPA can interact with Glu 350 (Figure 1).

In our previous work, we have designed and synthesized a series of *L*-isoserine derivatives as APN inhibitors and found that the tripeptide derivative **14b** (Figure 2) was the most active compound[13]. In order to find better APN inhibitors, we used compound **14b** as the leading compound and modified it as follows (Figure 2): (i) we maintained the *L*-isoserine scaffold to chelate the zinc ion and the *L*-phenylalanine group was replaced by different amino acids; (ii) The *L*-leucine reside was substituted by different amino acids or organic amines in order to closely interact with the hydrophobic pocket of APN.

Material and methods

Chemistry

All the materials were purchased from commercial vendors and used without further purification unless otherwise specified. Solvents were dried over $CaCl_2$ or distilled

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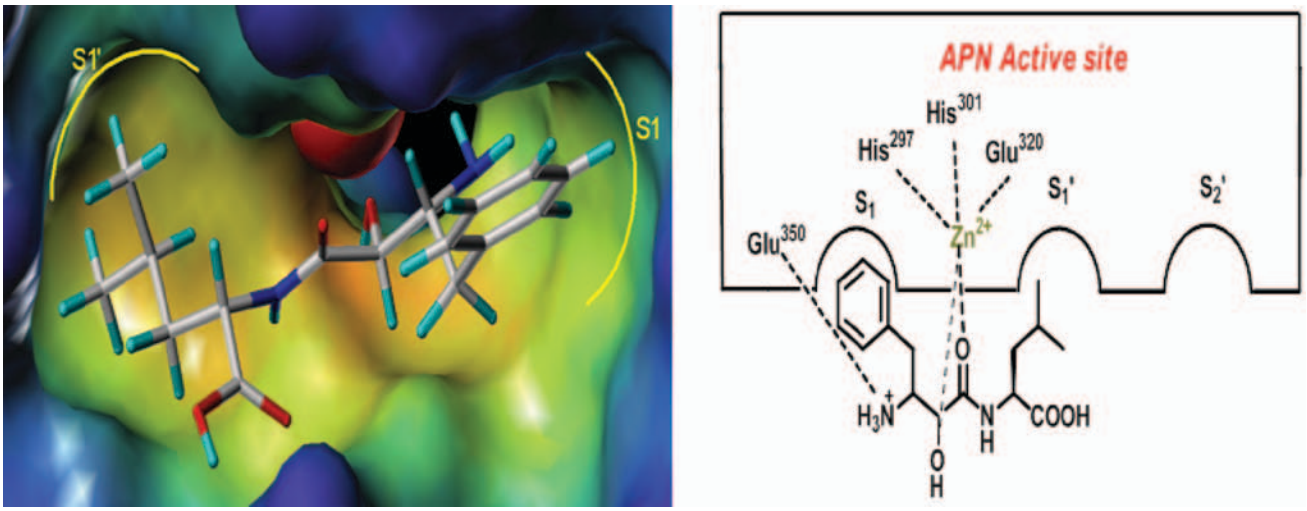


Figure 1. The binding character of **Bestatin** with the active site of APN.

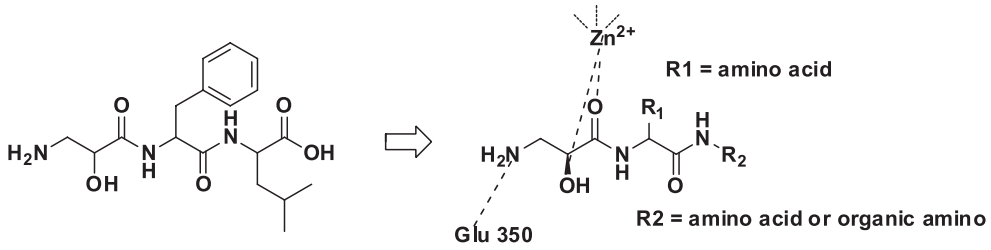


Figure 2. The strategy for the design of *L*-isoserine tripeptide derivatives as APN inhibitors.

prior to use and flash chromatography was performed using silica gel (200–300 mesh). All reactions were monitored by thin-layer chromatography on 0.25 mm silica gel plates (60GF-254) and visualize with UV light, or chloride ferric. Proton NMR spectra were determined on a Brucker DRX spectrometer (600 MHz). Measurements were made in DMSO-*d6* solutions. ESI-MS were determined on an API 4000 spectrometer. Melting points were conducted on electrothermal melting point apparatus (uncorrected). The final compound was purified by reversed-phase chromatography to give the desired compound.

General procedure for the synthesis of 2a–2e and 4a–4f

The title compounds amino acid methyl ester **2a–2e** were prepared from natural amino acids according to methods described in the literatures[14]. With the same materials, the title compounds Boc-protected amino acids **4a–4f** were prepared.

General procedure for the synthesis of 8a–8n

A 150 mL solution of compound **2a** (1.51 g, 8.3 mmol) in dried DCM was gently cooled to 0°C in ice bath. To the solution, TEA (1.72 mL, 24.75 mmol) was added dropwise. After compound **2a** was dissolved, HOBt (1.22 g, 9.0 mmol), DMAP (0.18 g, 1.5 mmol), **4a** (2 g, 7.5 mmol) and TEA (1.72 mL, 24.75 mmol) were added. The mixture was stirred for 15 min when EDCI (2.87 g, 15 mmol) was added in batches. The reaction mixture was stirred in ice bath for 2 h and then changed to room temperature for 12 h. The solvent was concentrated under vacuum to leave residue. The residue was redissolved with EtOAc (100 mL). The solution was washed with 1 mol/L citric acid solution, saturated $NaHCO_3$, water and saturated sodium chloride solution, dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by flash column chromatography (PE/EtOAc, 3:1(V/V)) to give the desired compound **6a** as a white solid (2.6 g), yield: 89.6%, mp. 95–98°C. ^{1}H NMR (DMSO-d_6) δ 7.929 (d, J = 7.8 Hz, 1H), 7.292-7.214 (m, 5H), 5.942 (d, 1H), 4.927-4.915 (m, J = 5.5 Hz, 1H), 4.220-4.208 (m, 1H), 3.678 (s, 3H), 3.452-3.438 (m, 1H), 3.212-3.189 (m, 1H), 2.538-3.529 (m, 1H), 1.570-1.552 (m, 2H), 1.428 (s, 9H), 1.121-1.110 (d, 3H), 1.101-0.898 (m, 3H); ESI-MS *m/z*: $[M+H]^+$ 393.5.

To the dried compound **6a** (2.6 g, 6.63 mmol), once in a while, a solution of EtOAc (15 mL) saturated with dry HCl gas was added. The reaction was stirred at room temperature for 4 h before being concentrated *in vacuo*. The residue was recrystalized with MeOH and ether to give **8a** (1.74 g) as a white crystal, yield: 79.96%, ^{1}H NMR (DMSO-*d6*) δ 8.034 (d, J = 7.8 Hz, 1H), 8.012 (s, 3H), 7.289-7.211 (m, 5H), 4.917-4.905 (m, 1H), 4.210-4.188 (m, 1H), 3.669 (s, 3H), 3.442-3.429 (m, 1H), 3.202-3.172 (m, 1H), 2.542-3.529 (m, 1H), 1.564-1.532 (m, 2H), 1.121-1.110 (d, 3H) 1.101-0.898 (m, 3H); ESI-MS *m/z*: $[M+H]^+$ 329.2.

General procedure for the synthesis of 9a–9c

Compound **5a** (0.62 g, 4.5 mmol) was dissolved in 100 mL of dried DCM. The solution was gently cooled to 0°C in ice bath. Then HOBt (0.73 g, 5.4 mmol), DMAP (0.1 g, 0.9 mmol) and compound **4a** (1.2 g, 4.5 mmol) were added. The mixture was stirred for 15 min when EDCI (1.72 g, 9 mmol) was added in batches. The reaction mixture was stirred in 0°C for 2 h and then changed to room temperature for 12 h. The solvent was concentrated under vacuum to leave residue. The residue was redissolved with EtOAc (100 mL). The solution was washed with 1 mol/L citric acid solution, saturated $NaHCO_3$, water and saturated sodium chloride solution, dried over $MgSO_4$ and evaporate *in vacuo*. The residue was purified by flash column chromatography (PE/EtOAc, 8:1(V/V)) to give the desired compound **7a** as a white solid (1.6 g), yield: 92.5%. ^{1}H NMR (DMSO-d_6) δ 8.403 (m, J = 7.8 Hz, 1H), 8.032 (dd, 1H), 7.401-7.271 (m, 5H), 7.251(m, 2H), 6.871 (m, 2H), 4.442-4.429 (m, 1H), 4.202-4.172 (m, 1H), 3.843 (s, 3H), 3.212-3.188 (m, 2H), 1.381 (s, 9H); ESI-MS *m/z*: $[M+H]^+$ 385.2.

To the dried compound **7a** (1.2 g, 3.75 mmol) was added once in a while a solution of EtOAc (50 mL) saturated with dry HCl gas. The reaction was stirred at room temperature for 4 h before being concentrated *in vacuo*. The residue was recrystalized with MeOH and ether to give **9a** as a white crystal (1.2 g), yield: 90.2%, ^{1}H NMR (DMSO-d_6) δ 8.543 (m, J = 7.8 Hz, 1H), 8.031 (m, 1H), 7.412-7.271 (m, 5H), 7.254 (d, 2H), 6.881 (d, 2H), 4.420-4.329 (m, 2H), 3.950 (m, 1H), 3.843 (s, 3H), 4.402-3.172 (m, 1H), 3.402-3.172 (m, 1H); ESI-MS *m/z*: $[M+H]^+$ 321.1.

General procedure for the synthesis of Boc-isoserine 11

The starting material *L*-isoserine is a white solid, (mp. 199–201°C, $[a]^{25}D = -32.5$ (c 1, H_2O)), was purchased from Shanghai Nuotai Chemical Co., Ltd, China. Compound **11** was synthesized following the general procedure as described above (preparation of **4a**). A colourless solid, yield: 92%, mp. 85–88°C; $[a]^{25}D = +6.7$(c 1, MeOH)), ^{1}H NMR (DMSO-*d6*) δ 7.934 (d, J = 7.8 Hz, 1H), 6.540 (s, 1H), 4.674–4.651 (m, 1H), 3.457 (dd, J = 13.80 Hz, J = 8.4 Hz, 1H), 3.214 (dd, J = 13.80 Hz, J = 8.4 Hz, 1H), 1.435(s, 9H); ESI-MS *m/z*: $[M+H]^+$ 206.1.

General procedure for the synthesis of 16a–16n

Compound **12a** was synthesized from N-protected *L*-isoserine (**11**) and *L*-phenylalanyl-*L*-isoleucine methyl ester hydrochloride (**8a**) following the general procedure as described above (preparation of **6a**), yield: 68%.

The solution of compound **12a** (1.4 g, 2.97 mmol) in MeOH at 0°C was added dropwise a solution of 1 mol/L LiOH (6 mL). The reaction was warmed to room temperature and stirred for 5 h. The solvent was concentrated under vacuum to remove the MeOH. The remaining mixture was added water (5 mL) and acidified to pH 1–2 by a 1 mol/L citric acid solution. The precipitate was collected, washed with water (10 mL) and dried overnight to give the crude product **14a** (0.68 g), yield: 50.5%. Compound **16a** was obtained through the deprotection of compound **14a** in the saturated HCl/EtOAc solution as the preparation of **8a**. The crude compound **16a** was purified by reversed-phase chromatography to give the desired compound **16a** as a white solid, yield: 50%. mp. 200–201°C, ^{1}H NMR (DMSO-d_6) δ 12.560 (s, J = 7.8 Hz, 1H), 8.184 (d, J = 7.8 Hz, 1H), 8.117 (s, 2H), 7.776 (d, J = 7.8 Hz, 1H), 7.265-7.176 (m, 5H), 6.518 (s, 1H), 4.468-4.461 (m, 1H), 4.013-3.939 (m, 1H), 4.011-3.929 (m, 1H), 3.047 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.984 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.889-2.872 (m, 1H), 2.765-2.674 (m, 1H), 1.169-1.235 (m, 3H),0.877-0.865 (m, 3H), 0.852-0.802 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 366.3.

The compounds **16b–16n** were synthesized following the general procedure as described above (preparation of **16a**).

General procedure for the synthesis of *L*-isoserine-*L*-phenylalanine-*L*-valine (16b)

A white solid, yield: 53.6%, mp. 190–191°C, ^{1}H NMR (DMSO-d_6) δ 12.674 (s, J = 7.8 Hz, 1H), 8.278 (d, J = 7.8 Hz, 1H), 8.119 (s, 2H), 7.756 (d, J = 7.8 Hz, 1H), 7.275-7.266 (m, 5H), 6.498 (s, 1H), 4.458-4.432 (m, 1H), 4.214-3.839 (m, 1H), 4.021-3.897 (m, 1H), 3.111 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.879 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.876-2.858 (m, 1H), 2.775-2.671 (m, 1H), 2.169 (m, 1H), 0.868-0.846 (m, 3H), 0.849-0.832 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 352.4.

General procedure for the synthesis of *L*-isoserine-*L*-phenylglycine-*L*-valine (16c)

A white solid, yield: 50.0%, mp. 139–140°C, ^{1}H NMR (DMSO-d_6) δ 12.554 (s, J = 7.8 Hz, 1H), 8.372 (d, J = 7.8 Hz, 1H), 8.329 (s, 2H), 8.303 (d, J = 7.8 Hz, 1H), 7.269-7.256 (m, 5H), 6.501 (s, 1H), 4.468-4.432 (m, 1H), 4.209-3.832 (m, 1H), 4.121-3.997 (m, 1H), 3.231 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.979 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.254 (m, 1H), 0.875-0.856 (m, 3H), 0.852-0.8421 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 338.4.

General procedure for the synthesis of *L*-isoserine-*L*-valine-*L*-phenylalanine (16d)

A white solid, yield: 52.1%, mp. 210–212°C, ^{1}H NMR (DMSO-d_6) δ 12.598 (s, J = 7.8 Hz, 1H), 8.267 (d, J = 7.8 Hz, 1H), 8.219 (s, 2H), 7.743 (d, J = 7.8 Hz, 1H), 7.310 -7.275 (m, 5H), 6.520 (s, 1H), 4.449-4.429 (m, 1H), 4.219-3.939 (m, 1H), 4.111 -3.893 (m, 1H), 3.099 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.902 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.869-2.849 (m, 1H), 2.768-2.688 (m, 1H), 2.321 (m, 1H), 0.897-0.886 (m, 3H), 0.859-0.842 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 352.2.

General procedure for the synthesis of *L*-isoserine-*L*-tyrosinase-*L*-valine (16e)

A pink solid, yield: 45.6%, mp. 100–104°C, ^{1}H NMR (DMSO-d_6) δ 12.589 (s, J = 7.8 Hz, 1H), 8.310 (d, J = 7.8 Hz, 1H), 8.219 (s, 2H), 8.012 (s, 1H), 7.749 (d, J = 7.8 Hz, 1H), 6.95 (s, 2H), 6.68(s, 2H), 6.500 (s, 1H), 4.449-4.438 (m, 1H), 4.219 -3.798 (m, 1H), 4.051-3.997 (m, 1H), 3.091 (dd, J = 13.8 Hz, J = 8.4 Hz, 1H), 2.881 (dd, J = 13.8 Hz, J = 8.4 Hz,

1H), 2.881-2.862 (m, 1H), 2.769-2.723 (m, 1H), 2.312 (m, 1H), 0.871-0.856 (m, 3H), 0.839-0.822 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 368.2.

General procedure for the synthesis of *L*-isoserine-*L*-valine-*L*-valine (16f)

A white solid, yield: 55.0%, mp. 181–182°C, ^{1}H NMR (DMSO-d_6) δ12.600 (s, *J* = 7.8 Hz, 1H), 8.391 (d, *J* = 7.8 Hz, 1H), 8.331 (s, 2H), 8.298 (d, *J* = 7.8 Hz, 1H), 6.499 (s, 1H), 4.471 -4.443 (m, 1H), 4.211-3.732 (m, 1H), 4.119-3.987 (m, 1H), 3.239 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 3.011 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.286 (m, 1H), 2.262 (m, 1H), 0.885-0.867 (m, 3H), 0.880-0.846 (m, 3H), 0.862-0.821(d, 3H), 0.858-0.842(d, 3H); ESI-MS *m/z*: $[M+H]^+$ 304.4.

General procedure for the synthesis of *L*-isoserine-*L*-phenylglycine-*L*-phenylalanine (16g)

A white solid, yield: 48.1%, mp. 196–198°C, ^{1}H NMR (DMSO-d_6) δ 12.598 (s, *J* = 7.8 Hz, 1H), 8.267 (d, *J* = 7.8 Hz, 1H), 8.219 (s, 2H), 7.743 (d, *J* = 7.8 Hz, 1H), 7.310 -7.275 (m, 5H), 7.026-7.014 (m, 5H), 6.520 (s, 1H), 4.449-4.429 (m, 1H), 4.219-3.939 (m, 1H), 4.111 -3.893 (m, 1H), 3.099 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.902 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.869-2.849 (m, 1H), 2.768-2.688 (m, 1H); ESI-MS *m/z*: $[M+H]^+$ 386.2.

General procedure for the synthesis of *L*-isoserine-*L*-valine- *L*-leucyl (16h)

A white solid, yield: 55.0%, mp. 182–183°C, ^{1}H NMR (DMSO-d_6) δ 12.588 (s, *J* = 7.8 Hz, 1H), 8.389 (d, *J* = 7.8 Hz, 1H), 8.312 (s, 2H), 8.310 (d, *J* = 7.8 Hz, 1H), 6.510 (s, 1H), 4.469 -4.439 (m, 1H), 4.199 -3.832 (m, 1H), 4.219-4.001 (m, 1H), 3.301 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 3.021 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.290 (m, 1H), 2.259 (m, 1H), 1.499 -1.435 (m, 3H), 0.895-0.877 (m, 3H), 0.889-0.856 (m, 3H), 0.872-0.831(d, 3H), 0.862-0.851 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 318.4.

General procedure for the synthesis of *L*-isoserine-*L*-leucyl -*L*-valine (16i)

A white solid, yield: 53.0%, mp. 181–182°C, ^{1}H NMR (DMSO-d_6) δ 12.579 (s, *J* = 7.8 Hz, 1H), 8.401 (d, *J* = 7.8 Hz, 1H), 8.308 (s, 2H), 8.309 (d, *J* = 7.8 Hz, 1H), 6.499 (s, 1H), 4.471 -4.439 (m, 1H), 4.211 -3.932 (m, 1H), 4.221-4.101 (m, 1H), 3.321 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 3.121 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.311 (m, 1H), 2.246 (m, 1H), 1.502 -1.445 (m, 3H), 0.898-0.887 (m, 3H), 0.891-0.876 (m, 3H), 0.868-0.842 (d, 3H), 0.871-0.841 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 318.4.

General procedure for the synthesis of *L*-isoserine-*L*-chloramphenicol-*L*-leucyl (16j)

A white solid, yield: 53.0%, mp. 180°C, ^{1}H NMR (DMSO-d_6) δ 12.579 (s, *J* = 7.8 Hz, 1H), 8.168-8.153 (d, *J* = 7.8 Hz, 2H), 7.868-7.671 (d, 2H), 5.254-5.249 (s, 1H), 4.485-4.480 (s, 1H), 4.053 -4.036 (m, 1H), 3.978-3.960 (m, 1H), 2.908-2.881 (m, 1H), 2.760-2.501 (m, 1H), 1.507-1.496 (d, 2H), 1.429-1.400 (m, 1H), 0.823-0.797 (m, 6H); ESI-MS *m/z*: $[M+H]^+$ 442.2.

General procedure for the synthesis of *L*-isoserine-*L*-phenylglycine-*L*-leucyl (16k)

A white solid, yield: 49.8%, mp. 205–206°C, ^{1}H NMR (DMSO-d_6) δ 12.549 (s, *J* = 7.8 Hz, 1H), 8.391 (d, *J* = 7.8 Hz, 1H), 8.298 (s, 2H), 8.313 (d, *J* = 7.8 Hz, 1H), 7.278-7.250 (m, 5H), 6.511 (s, 1H), 4.471-4.442 (m, 1H), 4.213-3.932 (m, 1H), 4.221-3.997 (m, 1H), 3.242 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 3.012(dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 1.522-1.442 (m, 3H), 0.881-0.876 (m, 3H), 0.852-0.841(d, 3H); ESI-MS *m/z*: $[M+H]^+$ 352.4.

General procedure for the synthesis of *L*-isoserine-*L*-tyrosinase-*L*-leucyl (16l)

A pink solid, yield: 40.6%, mp. 104–106°C, ^{1}H NMR (DMSO-d_6) δ 12.599 (s, *J* = 7.8 Hz, 1H), 8.289 (d, *J* = 7.8 Hz, 1H), 8.223 (s, 2H), 8.011 (s, 1H), 7.759 (d, *J* = 7.8 Hz, 1H), 6.951 (s, 2H), 6.682(s, 2H), 6.495 (s, 1H), 4.455-4.439 (m, 1H), 4.229 -3.804 (m, 1H), 4.151-3.989 (m, 1H), 3.191 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.981 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.981-2.872 (m, 1H), 2.779-2.743 (m, 1H), 1.502-1.446 (m, 3H), 0.869-0.8660 (m, 3H), 0.840-0.812 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 382.5.

General procedure for the synthesis of *L*-isoserine-*L*-eucyl-*L*-tyrosinase (16m)

A pink solid, yield: 42.6%, mp. 129–131°C, ^{1}H NMR (DMSO-d_6) δ 12.612 (s, *J* = 7.8 Hz, 1H), 8.314 (d, *J* = 7.8 Hz, 1H), 8.123 (s, 2H), 8.023 (s, 1H), 7.859 (d, *J* = 7.8 Hz, 1H), 7.002 (s, 2H), 6.692(s, 2H), 6.512 (s, 1H), 4.465-4.449 (m, 1H), 4.234 -3.814 (m, 1H), 4.161-3.978 (m, 1H), 3.291 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 3.002(dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.981-2.792 (m, 1H), 2.783-2.763 (m, 1H), 1.522-1.486 (m, 3H), 0.872-0.856 (m, 3H), 0.845-0.822 (d, 3H); ESI-MS *m/z*: $[M+H]^+$ 382.5.

General procedure for the synthesis of *L*-isoserine-*L*-phenylalanine-*L*-phenylalanine (16n)

A white solid, yield: 45.1%, mp. 184–185°C, ^{1}H NMR (DMSO-d_6) δ12.588 (s, *J* = 7.8 Hz, 1H), 8.277 (d, *J* = 7.8 Hz, 1H), 8.223 (s, 2H), 7.750 (d, *J* = 7.8 Hz, 1H), 7.311 -7.285 (m, 5H), 7.036-7.024 (m, 5H), 6.519 (s, 1H), 4.454-4.432 (m, 1H), 4.221-3.949 (m, 1H), 4.121 -3.993 (m, 1H), 3.199 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.913 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.879-2.855 (m, 1H), 2.778-2.698 (m, 1H), 2.832-1.794 (m, 1H), 2.509-2.434 (m, 2H); ESI-MS *m/z*: $[M+H]^+$ 410.2.

General procedure for the synthesis of 15a–15c

Compound **15a** was synthesized firstly through the condensation reaction of N-protected *L*-isoserine(**11**) and *L*-phenylalanyl-4-methoxybenzylamine following the general procedure as described above (preparation of **6a**), and then deprotecting the Boc group in the saturated HCl/EtOAc solution as the preparation of **8a**, yield: 76.1%. mp. 180–182°C, ^{1}H NMR (DMSO-d_6) δ 8.597 (d, *J* = 7.8 Hz, 1H), 7.929-7.917 (s, 2H), 7.907 (d, *J* = 7.8 Hz, 1H), 7.246-7.111 (m, 5H), 6.875-6.863 (s, 2H), 6.520 (s, 1H), 6.483-6.476 (s, 2H), 4.462 (m, 2H), 4.468-4.461 (m, 1H), 4.013-3.939 (m, 1H), 4.245-4.170 (m, 2H), 3.733 (s, 3H), 3.047 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.984 (dd, *J* = 13.8 Hz,

J = 8.4 Hz, 1H), 2.812-2.798 (m, 1H), 2.510-2.439 (m, 1H); ESI-MS *m/z*: $[M+H]^+$ 372.3.

General procedure for the synthesis of *L*-isoserine-*L*-phenylglycine-methoxybenzylamine (15b)

A white solid, yield: 56.1%, mp. 148–150°C, ^{1}H NMR (DMSO-d_6) δ8.597 (d, *J* = 7.8 Hz, 1H), 7.929-7.917 (s, 2H), 7.907 (d, *J* = 7.8 Hz, 1H), 7.246-7.111 (m, 5H), 6.875-6.863 (s, 2H), 6.520 (s, 1H), 6.483-6.476 (s, 2H), 4.462 (m, 2H), 4.468-4.461 (m, 1H), 4.013-3.939 (m, 1H), 4.245-4.170 (m, 2H), 3.733 (s, 3H), 3.047 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.984 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H); ESI-MS *m/z*: $[M+H]^+$ 368.3.

General procedure for the synthesis of *L*-isoserine-*L*-phenylglycine-2-furfurylamine (15c)

A white solid, yield: 48.1%, mp. 150–152°C, ^{1}H NMR (DMSO-d_6) δ 8.892 (d, *J* = 7.8 Hz, 1H), 7.929-7.917 (s, 2H), 7.907 (d, *J* = 7.8 Hz, 1H), 7.65 (d, 1H), 7.246-7.111 (m, 5H), 6.46 (d, 1H), 6.26 (d, 1H), 6.875-6.863 (s, 2H), 6.520 (s, 1H), 4.462 (m, 2H), 4.468-4.461 (m, 1H), 4.013-3.939 (m, 1H), 3.047 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.984 (dd, *J* = 13.8 Hz, *J* = 8.4 Hz, 1H), 2.812-2.798 (m, 1H), 2.510-2.439 (m, 1H); ESI-MS *m/z*: $[M+H]^+$ 332.4.

In vitro APN inhibition assay

IC_{50} values against APN were determined as previously described and by using *L*-Leu-*p*-nitroanilide as a substrate and microsomal aminopeptidase from Porcine Kidney microsomes (Sigma) in 50 mM PBS (pH 7.2) or suspension of A549, ES-2, HL-60 in PBS (2×10^5/well) as the enzyme. The hydrolysis of the substrate was monitored by following the change in the absorbance measured at 405 nm with a plate reader (Varioskan, Thermo, USA). All the solutions of the inhibitors were prepared in the assay buffer, and the pH was adjusted to 7.5 by the addition of 0.1 M HCl or 0.1 M NaOH. All the inhibitors were preincubated with APN at 37°C. The assay mixture, which contained the inhibitor solution (concentration dependent on the inhibitor), the enzyme solution (4 mg/mL final concentration) or the cells suspension, and the assay buffer, was adjusted to 200 μL.

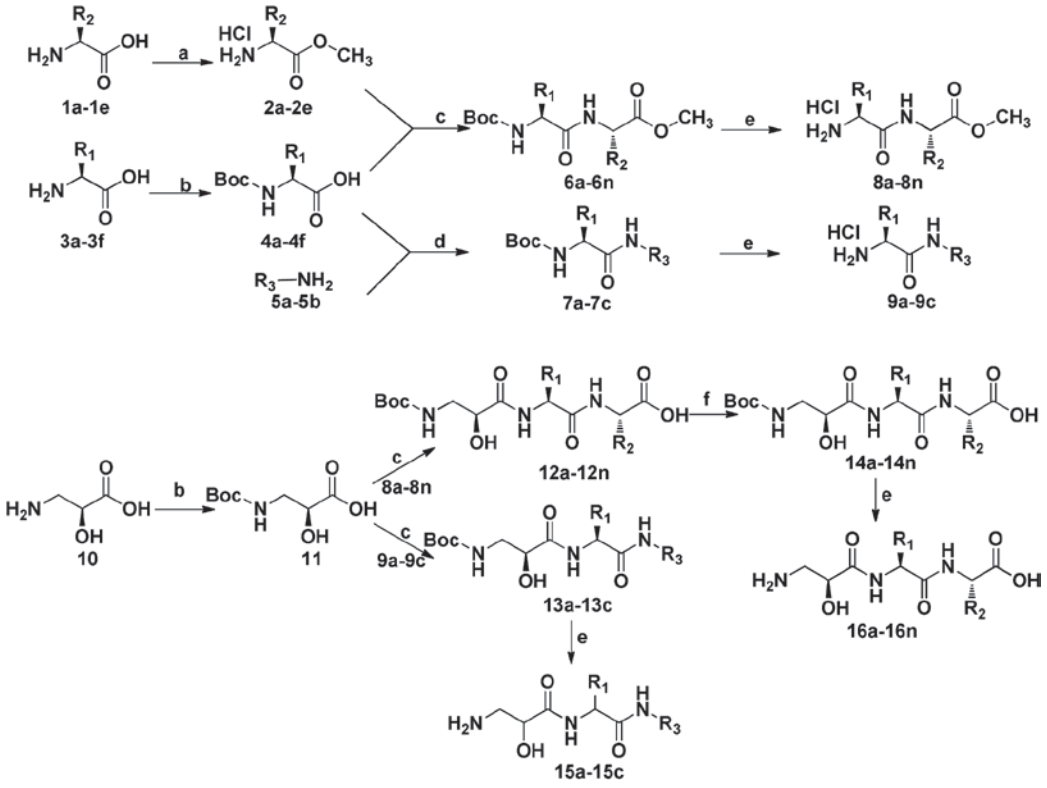
Antiproliferative activity assay

A549 cells, ES-2 cells (high APN expression) and MDA-MB-231 and K562 (low APN expression) were cultured in RPMI-1640 medium containing 10% FBS *in situ* at 37°C in 5% CO_2 humidified incubator. Cell proliferation was determined by MTT assay. In brief, cells were seeded in a 96-well plate (5×10^3/well for adherent cells and 1×10^4/well for suspension cells) and cultured for 4 h, followed by the addition of different concentrate of inhibitors in medium. After another 44 h, 0.5% MTT (5 mg/mL) solution was added to form the formazan product. At last, DMSO was used to solve the formazan and concentrations were monitored by detecting OD values at 570 nm through a plate reader (Varioskan, Thermo, USA).

Results and discussion

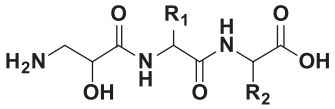
Chemistry

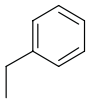
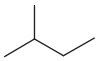
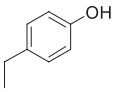
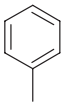
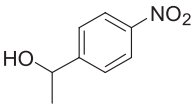
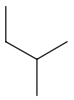
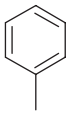
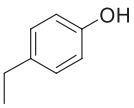
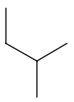
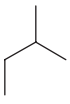
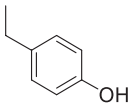
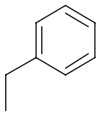
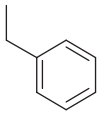
The target compounds were synthesized efficiently following the procedures as shown in Scheme 1. Amino



Scheme 1. Reagents and conditions: (a) MeOH, concentrated HCl; (b) 1 M NaOH, MeOH, $(Boc)_2O$; (c) HOBT/EDCI/DMAP/Et_3N, anhydrous CH_2Cl_2; (d) HOBT/EDCI/DMAP, anhydrous CH_2Cl_2; (e) concentrated HCl/MeOH; (f) MeOH, 1 M LiOH.

Table 1. The structures and inhibitory activities of series 1 compounds and **Bestatin** against APN.



Compd.	R1	R2	APN/IC_{50}[a] (μM) Porcine kidney	A549	ES-2	HL-60
16a			30.4 ± 2.1	5460 ± 55	ND[c]	ND[c]
16b			94 ± 4.6	80 ± 6.1	ND[c]	ND[c]
16c			51.5 ± 3.1	ND[c]	ND[c]	ND[c]
16d			30.2 ± 1.9	50 ± 3.1	ND[c]	ND[c]
16e			11.5 ± 0.9	460 ± 4.6	460 ± 5.1	ND[c]
16f			13.6 ± 1.1	430 ± 4.1	ND[c]	ND[c]
16g			5.8 ± 0.5	500 ± 5.1	560 ± 6.4	ND[c]
16h			6.5 ± 0.2	60 ± 1.1	200 ± 2.1	5350 ± 45
16i			5.16 ± 0.3	1820 ± 50	630 ± 3.6	2000 ± 23
16j			NI[b]	>10000	1550 ± 13	ND[c]
16k			11.6 ± 1.1	80 ± 7.1	300 ± 8.1	ND[c]
16l			2.51 ± 0.2	130 ± 8.2	380 ± 7.6	6100 ± 35
16m			17.5 ± 2.1	1040 ± 5.7	ND[c]	ND[c]
16n			6.5 ± 1.3	3140 ± 30	ND[c]	ND[c]
Bestatin			6.25 ± 0.4	20 ± 2.1	18 ± 1.8	520 ± 22

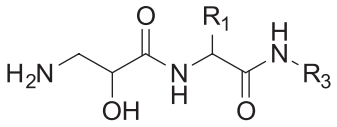
[a]All of the compounds were assayed three times, and their inhibition results are means of the three independent assays and expressed with standard deviations.
[b]NI, no inhibition.
[c]ND, not determined.

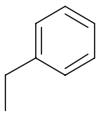
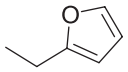
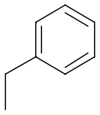
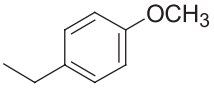
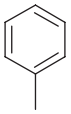
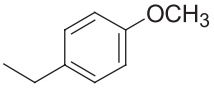
acids **1a–1e** were used as the starting materials to yield their methyl esters **2a–2e**, while amino acids **3a–3f** were protected by $(Boc)_2O$ to obtain **4a–4g**. Organic amides **5a–5b** were used without any protection. N-Boc-protected amino acids **4a–4f** were coupled with intermediates **2a–2e** and **5a–5b** by classical EDCI/HOBt method, and then converted into **8a–8o** and **9a–9c** by deprotecting the Boc group. Using the same procedure, Boc-protected *L*-isoserine **11** was coupled with intermediate **8a–8n** and **9a–9c** to yield tripeptide **12a–12n** and **13a–13c** containing protecting group. Finally, target compounds **16a–16n** were obtained by first reacting with based to deprotect methy group and then deprotecting Boc group while compounds **15a–15c** were get merely cleaving Boc group.

In vitro APN inhibition assay

All the target compounds were evaluated for their potential inhibitory activities against APN and the results were listed in Table 1 and Table 2. According to the data listed in Table 1, inhibitory effect could be detected among all the compounds except compound **16j**. Compound **16l** ($IC_{50} = 2.51 \pm 0.2$ μM) is the most potent inhibitor in all target compounds, which displayed inhibitory activity a little better than that of **Bestatin**($IC_{50} = 6.25 \pm 0.4$ μM), suggesting that

Table 2. The structures and inhibitory activity of the series 2 compounds against APN.



Compd.	R1	R3	APN/IC_{50}[a] (μM)	
			Porcine kidney	A549
15a			38 ± 3.6	890 ± 8.1
15b			24.4 ± 2.8	480 ± 6.6
15c			16.9 ± 1.5	510 ± 4.8

[a]All of the compounds were assayed three times, and their inhibition results are means of the three independent assays and expressed with standard deviations.

Table 3. Antiproliferative activities of the *L*-isoserine derivatives.

Compd	IC_{50}[a] (mM)			
	A549	MDA-MB-231	K562	HL-60
16a	>1000	4.34 ± 0.1	185 ± 9.1	ND[b]
16b	0.78 ± 0.04	80.3 ± 4.3	2.6 ± 0.11	ND[b]
16d	12.15 ± 0.3	0.9 ± 0.04	0.2 ± 0.06	0.24 ± 0.11
16e	0.97 ± 0.11	6.84 ± 1.1	4.3 ± 0.06	1084 ± 26
16f	1.90 ± 0.6	14.4 ± 1.2	>1000	104 ± 2.1
16g	3.67 ± 0.4	1.56 ± 0.02	483 ± 32	73 ± 7.1
16h	10.2 ± 0.7	10.9 ± 2.2	3.97 ± 0.5	9.3 ± 0.21
16i	31.5 ± 3.2	4.77 ± 0.08	10.6 ± 2.1	774 ± 8.8
16j	8.42 ± 0.9	>1000	842 ± 22	>1000
16k	1.68 ± 0.15	2.66 ± 0.06	3.83 ± 1.1	>1000
16l	>1000	615 ± 9.1	>1000	83 ± 2.1
16m	>1000	83 ± 2.1	24.5 ± 3.4	2.5 ± 0.21
16n	18 ± 0.41	203 ± 14	27.4 ± 4.1	ND[b]
15a	4.79 ± 0.1	0.3 ± 0.01	1.32 ± 0.05	0.3 ± 0.08
15b	2.7 ± 0.05	0.54 ± 0.12	0.27 ± 0.01	0.5 ± 0.01
15c	1.19 ± 0.21	0.7 ± 0.01	0.53 ± 0.11	0.88 ± 0.04
Bestatin	3.26 ± 0.02	2.36 ± 0.8	0.53 ± 0.21	0.43 ± 0.11

[a]All of the compounds were assayed three times, and their inhibition results are means of the three independent assays and expressed with standard deviations.
[b]ND, not determined.

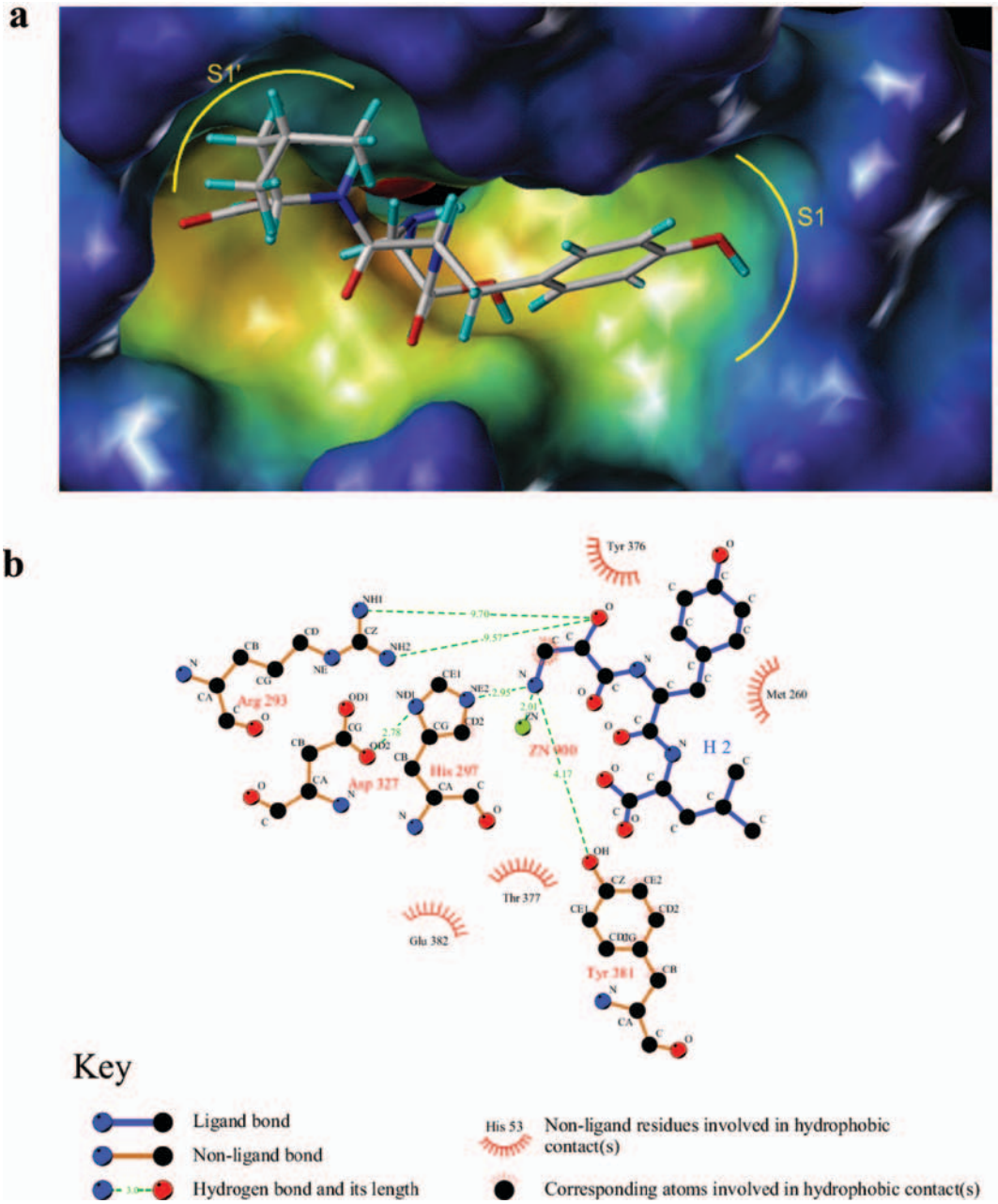


Figure 3. The docking result of compound **16l** with APN showed by LIGPLOT. (a) The docking result of **16l** with APN (**16l** is showed in capped sticks mode). (b) The docking result of compound **16l** is shown by LIGPLOT.

the tyrosine group may have something to do with hydrogen bond which would be helpful to increase the interaction with APN. On the other hand, the introduction of phenol group in the corresponding R2 site did not improved the activity appreciably as compound **16m** did not show outstanding inhibitory result ($IC_{50} = 17.5 \pm 2.1$ μM). It is possibly indicated that no hydrogen bond interaction is essential in this section. In addition, the phenyl group in R1 position would be much better than benzyl group to increase the activity as the phenylalanine derivatives (**16a**, **16b**, **16n**) show generally less inhibitory activity than the phenylqlycine analogues (**16c**, **16g**, **16k**). Series 2 compounds were designed and synthesized in order to determine the different influence of natural amino acids with arylamine. From the result of the enzymatic assay, we could see these compounds performed similar inhibitory activities with series 1 compounds. For instance, compounds **15a** ($IC_{50} = 38 \pm 3.6$ μM) and **15b** ($IC_{50} = 24.4 \pm 2.8$ μM) show similar activity with compound **16a** ($IC_{50} = 30.4 \pm 2.1$ μM) and **16b** ($IC_{50} = 94 \pm 4.6$ μM). In our case, despite the inhibitory activities of series 2 compounds maintained, the water-solubility reduced obviously.

In addition, the enzymatic inhibitory activity of all the compounds against APN was also determined with A549 (Human lung adenocarcinoma epithelial cell line) cells high-expressing APN. Results were shown in Table 1 and

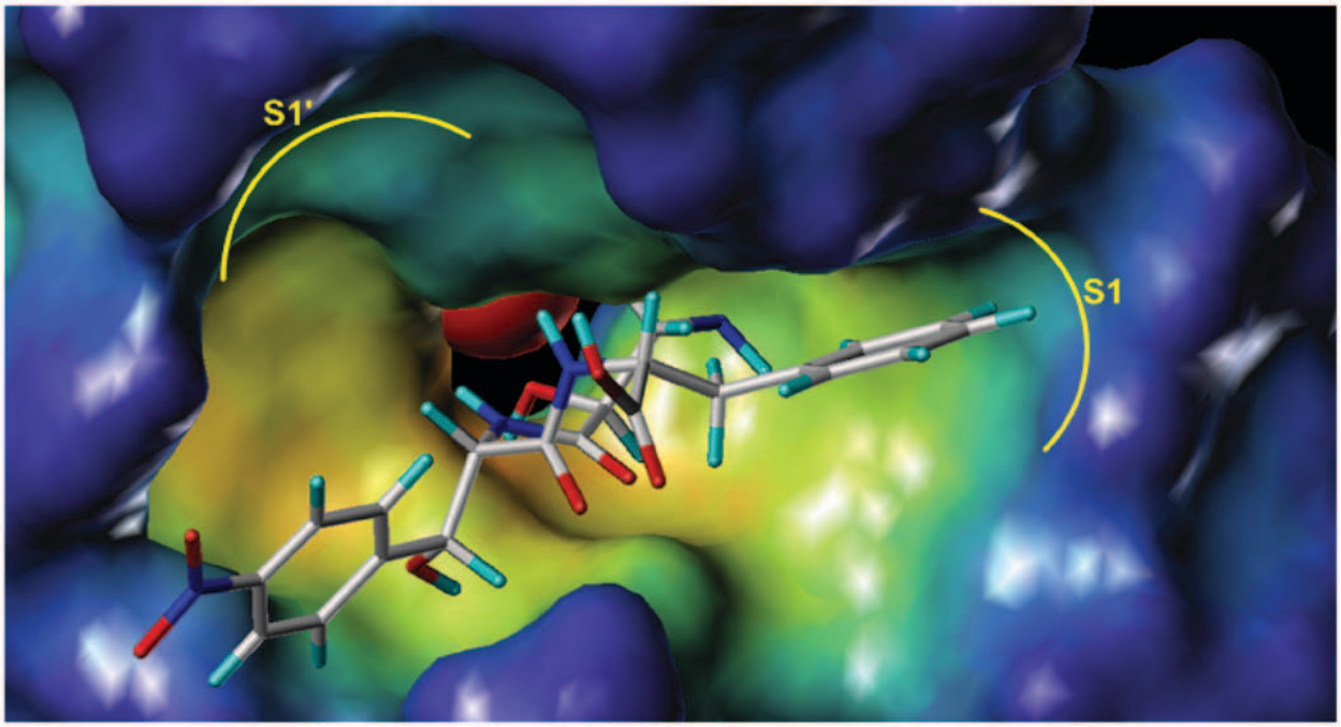


Figure 4. The docking result of **16j** with APN (**16j** is showed in capped sticks mode).

Table 2. From the results, we could learn that the inhibitory activity of these compounds on the cellar level was consistent with the assays on the enzymatic level in general. However, the previous proved compound **16l** (IC_{50} = 2.51 ± 0.2 μM) which was expected to perform the strongest effect against APN showed much weak activity against A549 cells while compound **16b** (IC_{50} = 94 ± 4.6 μM) and **16d** (30.2 ± 1.9 μM) showed better inhibitory activity on A549 cells. It was possibly due to the different binding characteristics of APNs among species. Moreover, compounds **16e**, **16g**, **16h**, **16i**, **16j**, **16k**, **16l** and **Bestatin** were evaluated for their activity against ES-2 (Ovarian Clear Cell Adenocarcinoma Cell Line) cells and the inhibitory activity of compounds **16h**, **16i**, **16l**, and **Bestatin** was also preformed on HL-60 (human promyelocytic leukemia cell line) cells. The results are shown in Table 1, from which we could tell that none of these compounds exhibited better activity than the control **Bestatin** and compound **16j**, which performed no activity in the enzymatic level, showed the weakest effect on ES-2 cells still.

Antiproliferative activity assay

On the other hand, all the compounds were detected for their potential effects on proliferation on four tumour cell lines (A549 cells, MDA-MB-231 cells, HL-60 cells and K562 cells) with **Bestatin** as the control via MTT assay. The results are shown in Table 3. It can be seen that some of the compounds showed similar even better anti-proliferation effect than **Bestatin** on different cell lines. However, compound **16l** does not exhibit outstanding anti-proliferation activity like the enzymatic assay. Besides, compound **16e**, **16g**, **16h**, **16i**, **16k** and **16l** which showed good activity in the APN inhibitory assay almost did not perform prominent anti-proliferation activity on the high-expressing APN cells (A549 cells and HL-60 cells) than the low-expressing APN cells (MDA-MB-231 cells and K562 cells), possibly implying a different mechanism from the interaction with APN in the respect of anti- proliferation activity of these compounds.

In order to investigate the interaction mode of *L*-isoserine derivatives with APN, the most active and inactive compound **16l** and **16j** was sketched and docked into the active site of APN (PDB code: 2DQM) using Surflex-Dock module of Sybyl 8.1. The result suggested that the binding mode of compound **16l** was similar with **Bestatin** and obvious gave the main reason for the distinct activity of both compounds. The zinc ion of APN was coordinating with the *L*-isoserine part of both compounds (Figures 3a and 4). In addition, the phenyl moiety of phenylalanine residue and tyrosine residue could plunge into the S1 pocket of APN (Figures 3a and 4). Besides, the leucine part of compound **16l** could deeply insert into the S1′pocket of APN (Figure 3a), while the chloramphenicol amine residue of compound **16j** was out of the S1′ pocket (Figure 4). Furthermore, compound **16l** also could form hydrogen bonds with Arg293, His297, and Try381 at the distance of 9.70, 2.95, and 4.17Å which

significantly contributed to improve the binding affinities (Figure 3b).

Conclusion

In summary, we designed and synthesized two series of novel *L*-isoserine tripeptide derivatives as APN inhibitors. The preliminary results showed that compounds **16h**, **16i**, **16g**, **16l** and **16n** were the most potent compounds and had similar inhibitory activity with **Bestatin**. The most effective compound **16l** (2.51 ± 0.2 μM), exhibited a little better enzymatic inhibitory activity against APN than control compound **Bestatin** (6.25 ± 0.4 μM).The SAR study of compound **16l** Clearly explain the excellent inhibitory activity. Compound **16l** could be used as a lead compound for further development of small molecular peptidomimetic APN inhibitors as new anticancer agents.

Declaration of interest

This work was supported by National High Technology Research and Development Program of China (863 project; Grant No. 2007AA02Z314), National Natural Foundation Research Grant (Grant No. 90713041) and The National Natural Science Foundation of China (Grant No. 21172134).

Reference

1. Fukasawa K, Fujii H, Saitoh Y, Koizumi K, Aozuka Y, Sekine K et al. Aminopeptidase N (APN/CD13) is selectively expressed in vascular endothelial cells and plays multiple roles in angiogenesis. Cancer Lett 2006;243:135–143.
2. Su L, Fang H, Yang K, Xu Y, Xu W. Design, synthesis and biological evaluation of novel *L*-lysine ureido derivatives as aminopeptidase N inhibitors. Bioorg Med Chem 2011;19:900–906.
3. Dixon J, Kaklamanis L, Turley H, Hickson ID, Leek RD, Harris AL et al. Expression of aminopeptidase-n (CD 13) in normal tissues and malignant neoplasms of epithelial and lymphoid origin. J Clin Pathol 1994;47:43–47.
4. Piela-Smith TH, Korn JH. Aminopeptidase N: a constitutive cell-surface protein on human dermal fibroblasts. Cell Immunol 1995;162:42–48.
5. Raynaud F, Bauvois B, Gerbaud P, Evain-Brion D. Characterization of specific proteases associated with the surface of human skin fibroblasts, and their modulation in pathology. J Cell Physiol 1992;151:378–385.
6. Zhang X, Xu W. Aminopeptidase N (APN/CD13) as a target for anti-cancer agent design. Curr Med Chem 2008;15:2850–2865.
7. Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A et al. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res 2000;60:722–727.
8. Aoyagi T, Yoshida S, Nakamura Y, Shigihara Y, Hamada M, Takeuchi T. Probestin, a new inhibitor of aminopeptidase M, produced by *Streptomyces azureus* MH663-2F6. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. J Antibiot 1990;43:143–148.
9. Rich DH, Moon BJ, Harbeson S. Inhibition of aminopeptidases by amastatin and bestatin derivatives. Effect of inhibitor structure on slow-binding processes. J Med Chem 1984;27:417–422.
10. Repic Lampret B, Kidric J, Kralj B, Vitale L, Pokorny M, Renko M. Lapstatin, a new aminopeptidase inhibitor produced by *Streptomyces rimosus*, inhibits autogenous aminopeptidases. Arch Microbiol 1999;171:397–404.
11. Chung MC, Lee HJ, Chun HK, Lee CH, Kim SI, Kho YH. Bestatin analogue from *Streptomyces neyagawaensis* SL-387. Biosci Biotechnol Biochem 1996;60:898–900.
12. Umezawa H, Aoyagi T, Suda H, Hamada M, Takeuchi T. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. J Antibiot 1976;29:97–99.
13. Yang K, Fen J, Fang H, Zhang L, Gong J, Xu W. Synthesis of a novel series of *L*-isoserine derivatives as aminopeptidase N inhibitors. J Enzyme Inhib Med Chem 2012;27:302–310.
14. Sunggak K, Jae IL, Youn CK. A simple and mild esterification method for carboxylic acids using mixed carboxylic-carbonic anhydrides. J Org Chem 1985;50:560–565.