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Novel aminopeptidase N inhibitors derived from antineoplaston AS2–5 (Part II)

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ABSTRACT

As our ongoing work, a series of peptidomimetic L-*iso*-glutamine derivatives derived from antineoplaston AS2–5 scaffold were prepared and their APN/CD13 and MMP-2 inhibitory activities were evaluated hereby. The results displayed that these compounds exhibited selective inhibition against APN as compared with MMP-2, with IC_{50} values in micromole range. Compounds **A1** and **A2** showed comparable APN inhibitory activities than the positive control bestatin.

1. Introduction

Satisfactory curative chemotherapeutic agents with high affinity and selectivity are expected to be rationally designed against specific tumor-associated targets. Given that aminopeptidase N (APN) is over-expressed on tumor cells and plays a pivotal role in tumor invasion, growth and angiogenesis,[1] it has been regarded as an attractive target for anticancer drugs design.

As a zinc-dependent endopeptidase, APN is also known as CD 13. It is a 150-kDa myeloid cell surface glycoprotein belonging to the M1 family, which preferentially releases neutral and basic amino acids from the N-terminal end of peptides.[2] It is noticeable that tumor cells which over-express APN, for example, melanoma cells, acute lymphocytic leukemic cells, as well as urological cancer cells, are highly motile and capable of migration through extracellular matrix.[3,4] Moreover, angiogenesis emanating from microvascular endothelial cells plays a central role in tumor growth, invasion and metastasis.[5] In case the mRNA of the APN/CD13 was knocked out, the level of angiogenesis decreased dramatically.[1] On the other hand, APN inhibitors (such as CD13 monoclonal antibodies or bestatin) could significantly block induced-retinal neovascularization in mice and in chorioallantoic membrane angiogenesis in vitro.[6,7] Accordingly, it is useful to develop potential APN inhibitors (APNIs) to block its enzymatic activity for medical and therapeutic utilization.[8]

In fact, many natural or small molecule inhibitors of APN/CD13 have been reported previously.[9–11] Amongst these inhibitors, bestatin, an antibiotic of microbial origin with APN inhibitory activity,[12] has become a useful tool as positive control in elucidating many physiological conditions.[13] Almost all these compounds are pseudodipeptides bearing zinc-chelating functionality. As a consequence, inhibitors containing zinc-binding groups (ZBGs) should be optimal selection in the designed molecules. This kind of inhibitors can interact with the catalytic zinc ions of zinc-dependent metalloenzymes and sequentially inhibit the metastatic spread of tumors and block the processes of tumor neovascularization.[14]

Recently, the 3D-structures of APN have been investigated by the X-ray crystallographic studies on the co-crystal of the enzyme and various inhibitors.[15,16] Accordingly, the interactions of bestatin with the active sites of APN can be illustrated by a simplified model (see Fig. 1). It revealed that beside the catalytic center zinc(II) ion of APN, there are two hydrophobic binding domains, which are called S_1 pocket and S_1' pocket, respectively. Therefore, one or more

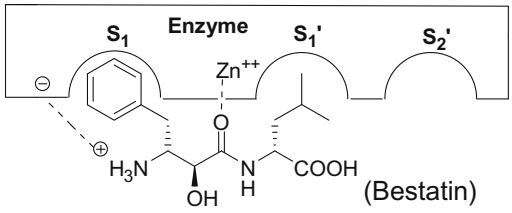

Figure 1. Schematic representation of the interaction of Bestatin with APN.

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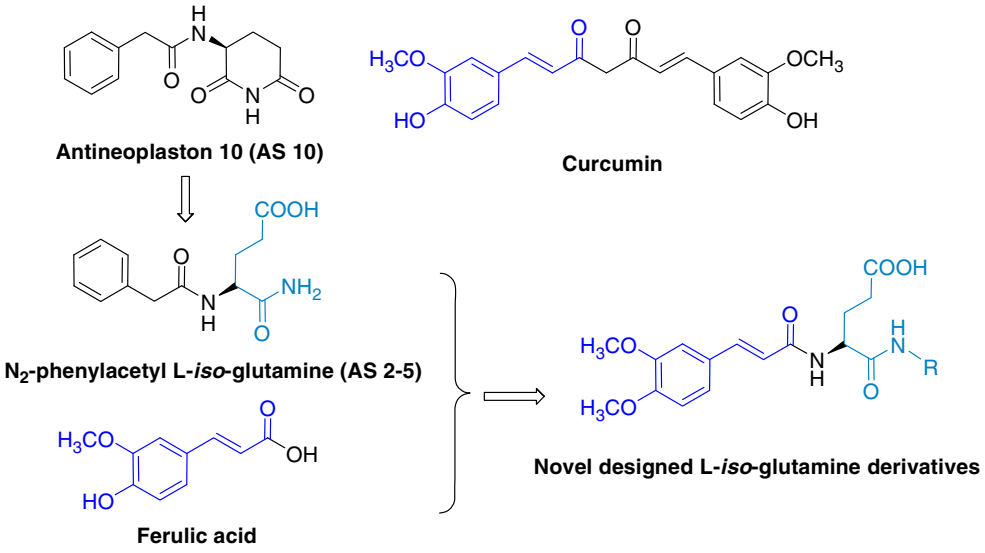


Figure 2. Chemical structures of ferulic acid, curcumin, AS-10, AS-25 and the newly designed compound.

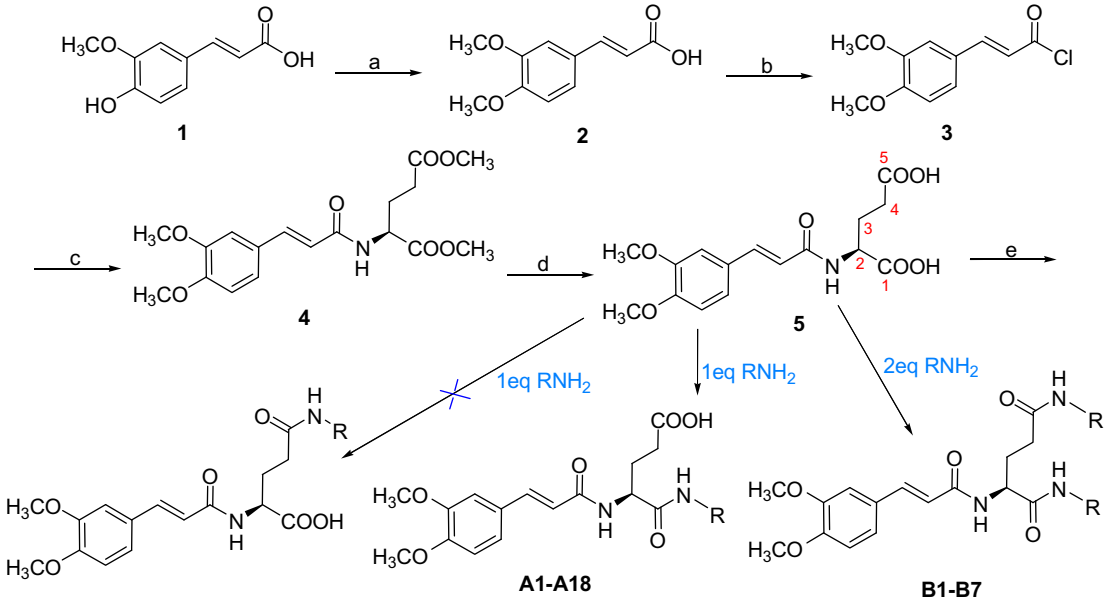
hydrophobic side chains should be introduced to insert into these pockets to generate effective interactions.

According to these findings, our group has previously reported a series of novel APNIs with remarkable APN inhibitory activities in the enzymatic assays.[17–20] In our ongoing efforts to identify more potent APNIs, we herein describe the synthesis and enzymatic evaluation of analogues derived from the antineoplaston AS2-5 (N_2-phenylacetyl L-*iso*-glutamine),[21,22] one of the active degradation product of antineoplaston A-10 (3-phenylacetylamino-2,6-piperidinedione, NSC-648539), which is known to be the first chemically identified antineoplaston with anti-tumor activity (see Fig. 2).[23,24]

Meanwhile, we noticed that phenolic (*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylic acid (ferulic acid; Fig. 2), one of efficient biologically component of Chinese traditional medicine herbs *Angelica sinensis*, *Rhizoma Chuanxiong*, and *Asafetida*, possess significant anti-oxidative and chemopreventive activities.[25,26] For instance, it was found that curcumin (see Fig. 2), a ferulic acid-based natural product, has shown irreversible APN inhibitory activity.[27] Hence, according to the 'combination principle', we therefore envision that the conjunction of antineoplaston AS-25 scaffold with ferulic acid fragment might generate a more efficacious scaffold for potential enzymatic activity of APN/CD13.

2. Chemistry

In order to study the SAR of these novel peptidomimetic compounds, different L-*iso*-glutamine derivatives were designed and synthesized via the route outlined in Scheme 1. Starting from commercially available ferulic acid **1**, the key intermediate (*S*,*E*)-2-(3-(3,4-dimethoxyphenyl)acrylamido)pentanedioic acid **5** was obtained via the sequence of methylation, acylation, nucleophilic



Scheme 1. Reagents and conditions: (a) Me_2SO_4, NaOH; (b) $SOCl_2$, C_6H_6; (c) L-dimethyl 2-aminopentanedioate, CH_2Cl_2; (d) Na_2CO_3, H_2O; (e) RNH_2 (1 equiv, for A1–A18; 2 equiv, for B1–B7), DCC, $CHCl_3$.

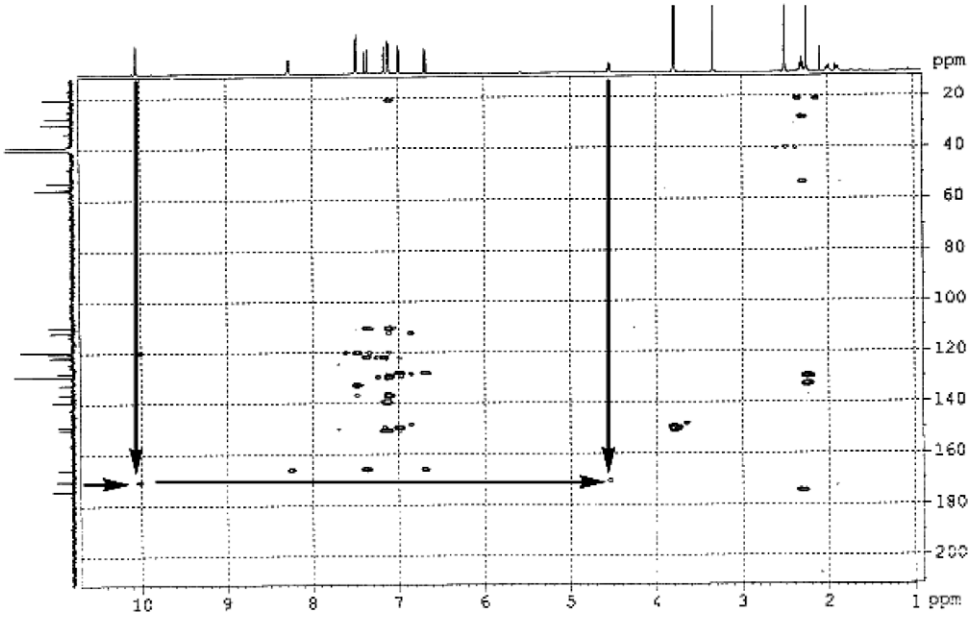


Figure 3. HMBC spectrum of compound **A1**.

substitution, and dehydration. This was followed by coupling with various primary amines (1 equiv and 2 equiv, respectively) in the presence of DCC to afford the primary amide peptidomimetics **A1–A18** and **B1–B7**.

3. Results and discussion

3.1. Structure confirmation for series A compounds A1–A18

As shown in Scheme 1, theoretically speaking, when compound **5** was treated with 1 equiv of nucleophilic agents, both C5 and C1 center might be attacked to produce mixture compounds. In fact, it revealed that only C1-based amide derivatives (**A1–A18**) were obtained, this might be due to the more adjacent to NH group of C1 in comparison with C5, resulting in lower electron cloud density. As a result, when the electronegative nitrogen atom of NH_2 attacks the electropositive carbon atom of a carbonyl group, it is inclined to attack C1 to get target compounds.

Take compound **A1** as an example, the corresponding chemical structure was further validated by IR, ^{1}H NMR, ^{13}C NMR, mass spectrometry (MS) and HMBC spectrum. Figure 3 diagrammed the HMBC spectrum (DMSO-d_6, 500 MHZ, ppm) of **A1**. There are cross-peaks between N5–H (10.04) and C4 (170.8), C3–H (4.54) and C4 (170.8), N2–H (8.27) and C1 (165.3), as well as C7–H (2.29) and C8 (173.1). Whilst N5–H (10.04) and C8 (dissociative carboxyl 173.1) have no correlative cross-peak. This information indicated that the NH_2 moiety was attacked at the C4 position, the dissociative carboxyl group was at the C8 position and, structures of compounds **A1–A18** were confirmed to be correct. Moreover, this conclusion could be elucidated as Figure 4, which has been shown by arrows. Besides, the IR, ESI-MS analytical and ^{1}H NMR data of other target compounds were in full agreement with the proposed structures (see Section 5).

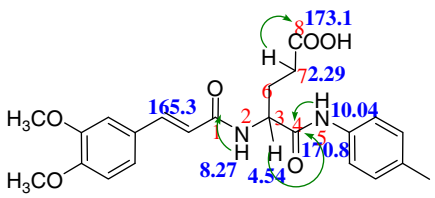


Figure 4. Diagram of the main correlative relations of **A1**.

3.2. Structure–activity relationship studies (in vitro) for series A compounds

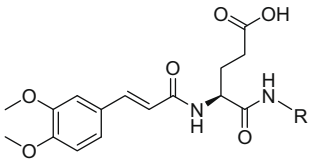
The target compounds were evaluated for their inhibitory activities toward APN/CD13 and MMP-2. Similar to APN, MMP-2 is also a zinc-dependent metalloproteinase involved in the process of tumor invasion and metastasis.[28,29] Thereby the assay was performed on both of APN and MMP-2 so as to identify their selectivity and, all the inhibition results are summarized in Tables 1 and 2. And else, bestatin was used as the positive control.

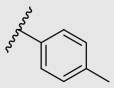
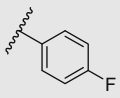
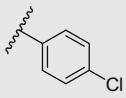
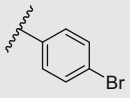
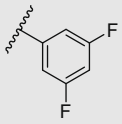
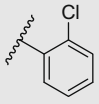
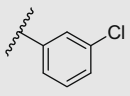
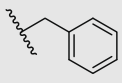
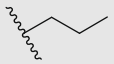
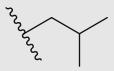
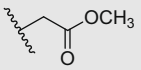
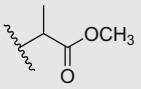
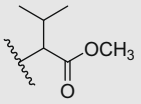
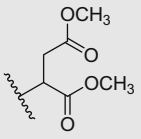
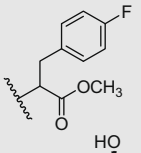
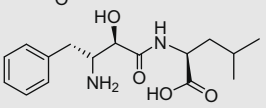
As shown in Table 1, it is worthy to note that these mono-substituted amide derivatives (**A1–A18**) displayed a better enzymatic inhibition towards APN as compared with MMP-2, with IC_{50} values lying in micromole level. The results, to a certain extent, validated our strategy for rational designing of potential APNIs. As the above mentioned selectivity against APN, the following SARs were mainly discussed about APN inhibition.

Among these inhibitors, generally speaking, compounds with aromatic side chain showed better activity compared to aliphatic derivatives. The possible reason may be due to the π system of the aromatic ring enhancing the interaction with the hydrophobic region of the enzyme.

Substitution on aromatic ring (**A1–8**) also has impact on bioactivity. Compound **A1**, with methyl-substitution at the *para*-position in the aromatic ring displayed the best APN inhibitory activity among series A compounds. Comparing compounds with halogen-substitution at the *para*-position in the aromatic ring (**A2–4**), it seems that the increased bulk of halogen substituents leads to impaired activity, suggesting there is a space requirement in the binding pockets to accommodate the suitable substituents. Additionally, mono-substitution of fluorine at the *para*-position in the aromatic ring (**A2**) displayed higher affinities (IC_{50} = 23.4 ± 3.2 μM) than at the *ortho*-position (**A6**, 56.8 ± 6.4 μM). For the chlorine counterparts, the *para*-substituted derivative (**A3**) gave the highest activity, the *ortho*-one (**A7**) was in

Table 1
The inhibitory activities of series A compounds



Compds	R	IC_{50} (μM)[a]	
		APN/CD13	MMP-2
A1		20.7 ± 0.6	37.8 ± 4.2
A2		23.4 ± 3.2	47.6 ± 0.8
A3		34.5 ± 3.8	59.9 ± 2.3
A4		58.1 ± 3.3	71.2 ± 0.3
A5		98.6 ± 4.3	116.3 ± 0.6
A6		56.8 ± 6.4	71.4 ± 5.1
A7		38.3 ± 4.2	64.3 ± 1.8
A8		80.5 ± 2.5	120.8 ± 4.8
A9		177.6 ± 7.9	235.8 ± 4.3
A10		125.8 ± 8.4	159.1 ± 6.6
A11		184.3 ± 5.7	211.4 ± 3.0
A12		237.5 ± 0.8	283.6 ± 8.4
A13		207.2 ± 6.6	256.1 ± 1.3
A14		50.4 ± 2.3	79.6 ± 3.0
A15		55.1 ± 1.8	83.2 ± 0.7
A16		69.2 ± 3.9	76.8 ± 4.5
A17		60.5 ± 2.6	101.5 ± 8.9
A18		43.7 ± 2.1	76.5 ± 0.9
Bestatin		13.1 ± 0.7	40.5 ± 1.2

[a] Values are means ± standard error of three experiments.

the next place, and the *meta*-one (**A8**) presented the least activity. On the other hand, di-substitution of fluorine at the 3-, 5-position of the aromatic ring exhibited impaired potency than at the 2-, or 4-position. However, the introduction of benzyl moiety (**A9**) caused obviously decreased potency.

When it comes to compounds with aliphatic side chain (**A10–13**), to some extent, length of R groups was negatively relative with the APN inhibition, suggesting it is unfavorable to increase the length of substituents along the orientation of the R groups. This might be due to longer groups were unfavorable to the accommodation with the enzymes' hydrophobic domains.

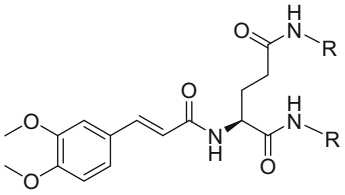
Comparing **A14–18**, the introduction of carbomethoxy residue ($COOCH_3$) as a ZBG produced improved potency. As ZBGs, both carbomethoxy and its corresponding metabolite carboxylic acid (COOH) can chelate with zinc at the catalytic center of the enzyme, resulting in significantly enzymatic inhibition.

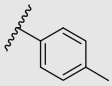
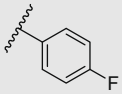
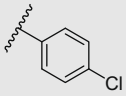
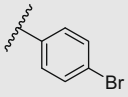
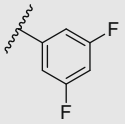
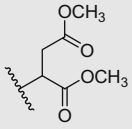
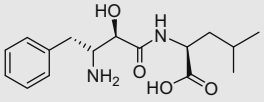
Finally, although compounds **A1** and **A2** gave comparable APN inhibitory activities with bestatin, all of the tested compounds demonstrated weaker potency in comparison with the positive control. Therefore, further structural optimization should be attentively investigated towards series A.

3.3. Structure–activity relationship studies (in vitro) for series B compounds

Overall, the SAR patterns of B series (**B1–B7**) were similar to those of A series (**A1–A18**), as shown in Table 2. However, these di-substituted amide derivatives displayed obviously weaker APN inhibition as compared with mono-substituted ones. Thereby, except compounds listed in Table 2, no further chemical modification was conducted towards series B. The possible reason might be that too bulky side chains might lead to the difficulties to accommodate the active domain of APN. For example, disubstitution of fluoro

Table 2
The inhibitory activities of series B compounds



Compds	R	IC_{50} (μM)[a]	
		APN/CD13	MMP-2
B1		334.7 ± 7.2	397.6 ± 8.9
B2		371.3 ± 9.5	368.1 ± 11.3
B3		405.2 ± 15.7	470.6 ± 13.8
B4		577.6 ± 10.2	na [b]
B5		405.2 ± 12.4	470.7 ± 10.1
B6		na[b]	na[b]
B7		213.2 ± 7.6	340.8 ± 9.0
Bestatin		13.1 ± 0.7	40.5 ± 1.2

[a] Values are means ± standard error of three experiments.
[b] Not activity.

group at the 3-, 5-position of the aromatic ring exhibited sharply impaired potency than mono-substitution of fluorine atom at the 2-position, even has no APN or MMP-2 inhibitory potency. Or in other words, carboxyl group, as an essential ZBG, should be considered to be reserved when designing potent APNIs.

3.4. Binding mode

Compound **A1**, which showed the best affinity in our target compounds, was selected to study its binding mode with APN. Firstly, the predicted conformation of **A1** was optimized with the Powell Energetic Gradient method built in the Sketch/Build Edit model (SYBYL6.91, Linux 7.3).[30] As compared with the known APNI bestatin, the comparable results showed the affinity to the same spatial regions (S_1 and S_1'), implying the similar APN inhibitory activities. In precise words, both the isopropyl group of bestatin and the introduced toluene fragment of **A1** can adjust their flexibility to extend into the S_1' pocket with their preponderant conformations, as shown in Figure 5. In contrast, the similar situation can be seen between the phenyl group of bestatin and the di-methoxy phenyl portion of **A1**.

To further understand the APN inhibitory difference between series A and series B, the preferred pharmacophore docking studies were carried out via the FLEXX flexible-Dock program. The interactions of A1 and B1 with active site of *Escherichia coli* APN (PDB: 2DQM) were compared, which are shown in Figure 6. Not surprisingly, both the di-methoxy phenyl fragment and the introduced di-substituted amide portion of **B1** could not well-orienting interact with the active domain (S_1 and S_1') of APN. Unlike **B1**, the moderate bulk of mono-substituted amide portion of **A1** could undergo torsional motion to allow their suitable conformations to accommodate the S_1 and S_1' pockets, respectively.

Finally, the selective inhibition of target compounds might also be explained by the molecular docking method of the representative compounds **A1** and **B1** with the active site of MMP-2 (PDB ID: 1HOV). As diagrammed in Figure 7, both the two compounds could not occupy the S_1 and S_1' pockets of MMP-2, resulting weaker MMP-2 inhibitory activities. In addition, the comparable docking results also graphically validated the importance of carboxyl group (as a ZBG) when designing potent APNIs. This is due to the effective interaction of **A1** with the catalytic zinc ion 166 of MMP-2, while **B1** was far from it, leading to sharply decreased MMP-2 inhibition, consistent with enzymatic assay results. The binding mode information encouraged us to further design antineoplaston AS2-5 scaffold based APNIs.

It should be pointed out that although the computed informational assay totally supported our assumption, the exact binding model of the target compounds with APN should be verified from further X-ray crystal studies.

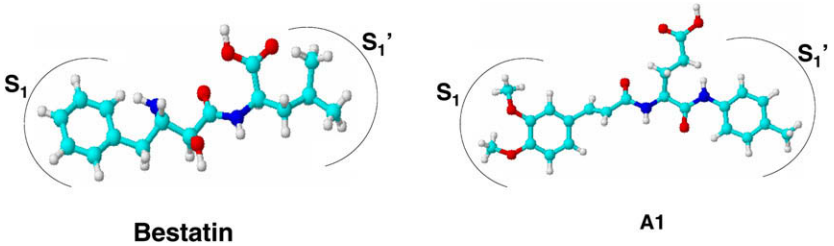


Figure 5. Comparison of preponderant conformations between bestatin and compound **A1**.

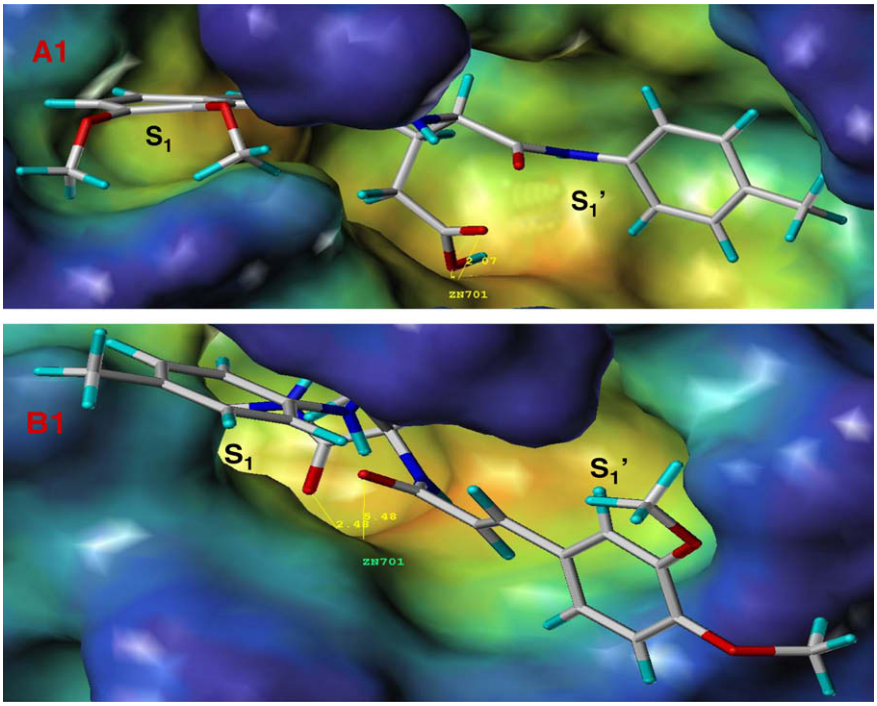


Figure 6. Comparison of FlexX docking results of compounds **A1** and **B1** with the active site of *E. coli* APN (PDB: 2DQM).

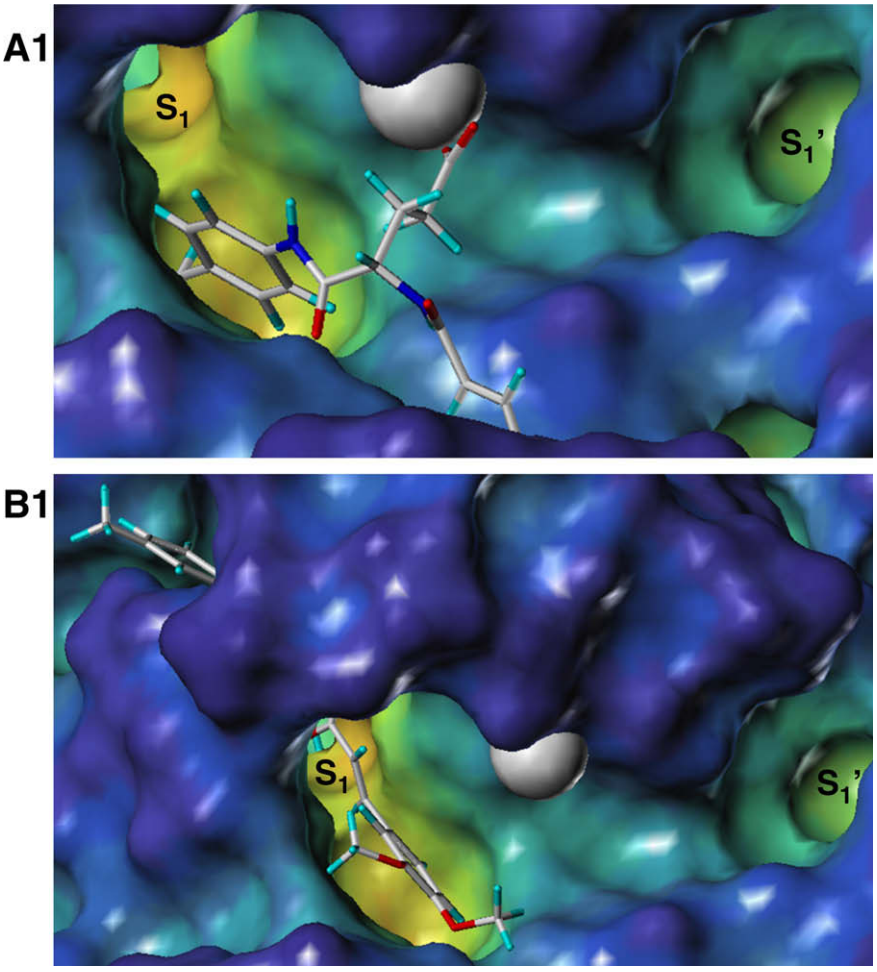


Figure 7. Comparison of FlexX docking results of compounds **A1** and **B1** with the active domain of MMP-2 (PDB ID: 1HOV).

4. Conclusion

In conclusion, we have developed two series of novel peptidomimetic L-*iso*-glutamine derivatives based on the antineoplaston AS2-5 scaffold. All of the compounds possess selective inhibitory activity against APN as compared with MMP-2. This feature may offer a critical point in designing selective metalloproteinase inhibitors so as to optimize the recognition of APN. Besides, further in vitro and in vivo evaluation of these compounds on anti-tumor activity is underway.

5. Experimental

5.1. General procedures

Silica gel for column chromatography (CC) and analytical thin-layer chromatography (TLC) plates precoated with Silica Gel GF_{254} were commercial available from Qingdao Haiyang Chemical Company, Qingdao, China. Reaction courses and product mixtures were routinely monitored by TLC on 0.25 mm Silica Gel 60 F_{254} plates and visualized under ultraviolet (UV) light (254 nm), or iodine (I_2) vapor. Flash-chromatography (FC) was performed using 200–300 mesh silica gel and the solvent system was indicated in the procedure. All solvents were of reagent grade and, when necessary, were purified and dried by standard methods. Melting points (Mp) were determined on an *X-6* micro-melting-point apparatus (*Beijing Tech Co., Ltd*) with no correction. Infrared spectra (IR) were recorded in the range of 4000–600 cm^{-1} using a *Nicolet Nexus 470FT-IR* spectrometer, and KBr disks were used as indicated. ^1H NMR spectra were determined on a *Brucker Avance DRX-600* spectrometer, with chemical shift (δ) given in ppm upfield from Me_4Si (TMS) as an internal standard, and coupling constants *J* were recorded in hertz (Hz). Peak multiplicities are abbreviated: single, s; double, d; triple, t; quartet, q; multiplet, m; broad, br. Electrospray ionization mass spectrometry (ESI-MS) was performed on an *API-4000* triple-stage quadrupole instrument. Anhydrous reactions were carried out in oven-dried glassware under a nitrogen atmosphere, and all anhydrous solvents were distilled over CaH_2 or Na/benzophenone prior to use. Yields refer to purified products and are not optimized.

5.2. Syntheses

5.2.1. (*E*)-3-(3,4-Dimethoxyphenyl)acrylic acid (2)

To a stirred solution of ferulic acid (**1**, 19.4 g, 100 mmol) in 60 ml of 4 N NaOH was added $(CH_3)_2SO_4$ (18.9 g, 150 mmol) dropwise, keeping the inside temperature under 20 °C. The resulting solution was allowed to stir at 20–30 °C for 20 min, another portion of $(CH_3)_2SO_4$ (150 mmol) and 40 ml of 4 N NaOH were successively added. The mixture was slowly heated to 90 °C and maintained at this temperature for 1 h. After refluxing for another 2 h, the reaction mixture was allowed to cool to rt. The solution was then acidified with 2 N HCl to pH 2, filtered, and the solid was washed twice with distilled water. The crude product was recrystallized with 70% EtOH to give compound **2** (17.5 g, 84.13%) as white needle crystals: Mp 179–181 °C. ^1H NMR δ 12.11 (s, 1H, COOH), 7.61 (Ar-CH), 6.77 (s, 1H, Ar-H), 6.65 (s, 1H, Ar-H), 6.45 (CH–COO), 3.83 (s, 6H, CH_3O–). ESI-MS: *m*/*z* $[M+H]^+$ 209.2 [M+1].

5.2.2. 3,4,5-Trimethoxybenzoic chloride (3)

To a stirred solution of **2** (20.8 g, 100 mmol) in benzene (250 ml) was added dropwise with $SOCl_2$ (40 ml, 548 mmol). The reaction mixture was refluxed for 4 h, and the solvent was removed to obtain compound **3** as pale yellow oil. Then another 50 ml of benzene was added and evaporated until the excess $SOCl_2$ was completely removed. The crude product was used immediately in the next reaction without further purification.

5.2.3. (*R*)-2-(3,4,5-Trimethoxybenzamido)pentanedioic acid (4)

Preparation of L-glutamine acid dimethylesters: L-glutamine acid (20 g) was added to a cooled solution of and anhydrous methanol (140 ml). Successively 16 ml of $SOCl_2$ was added at −5 °C in small portions over 1 h. The resulting suspension was allowed to stir overnight at room temperature. The insoluble material was filtered off and the filtrate was concentrated in vacuo. The following mixture was basified with $NH_3{\cdot}H_2O$ to pH 5–6, extracted twice with chloroform, then dried over anhydrous Na_2SO_4, filtered, and evaporated to give colorless oil (18.8 g), which was further used without further purification.

To a solution of L-glutamine acid dimethylesters (19.25 g, 110 mmol) in CH_2Cl_2 (100 ml) at −5 °C was added a solution of compound **3** in 100 ml CH_2Cl_2 in small portions. The mixture was stirred at this temperature until the solid has almost completely dissolved (about 1 h). The resulting residue was purified by flash chromatography using hexane/EA (4:1–1:1) as an eluant to yield compound **4** (23.9 g, 65.5%). Mp 151.0–152.5 °C, ESI-MS: *m*/*z* (rel intensity) 365.5 [M+1].

5.2.4. (*E*)-2-(3-(3,4-Dimethoxyphenyl)acrylamido)pentanedioic acid (5)

A suspension of compound **4** (18.25 g, 50 mmol) and 1 N Na_2CO_3 (100 ml) was stirred at 75–80 °C until the disappearance of the starting material and the clarification of the reaction mixture, checking via TLC. The resulting mixture was acidified with 1 N HCl to pH 2, extracted with EA (50 ml × 5), then dried over anhydrous Na_2SO_4, filtered, and evaporated the organic solvent. The obtained residue was dissolved by a small amount of acetone and allowed to place at rt. Collect the white columnar crystals on a Buchner funnel, then dried to give **5** (12.5 g, 82%). Mp 104.0–105.3 °C, ESI-MS: *m*/*z* (rel intensity) 337.3 [M+1].

5.2.5. (*S*,*E*)-5-(*p*-Toluidino)-4-(3-(3,4-dimethoxyphenyl)acrylamido)-5-oxopentanoic acid (A1)

To a solution of compound **5** (3.5 g, 10 mmol) in $CHCl_3$ (20 ml) at −5 °C was added a solution of 1,3-dicyclohexylcarbodiimide (DCC, 2.06 g, 1 equiv, 10 mmol) in 50 ml $CHCl_3$ in small portions. After completion the DCC, a solution of *p*-toluidine (1.07 g, 10 mmol) in 20 ml $CHCl_3$ was added dropwise. Then the mixture was stirred at this temperature until the solid has almost completely dissolved (about 5 h). The insoluble material (dicyclohexylurea, DCU) was filtered off and washed with cold 1 N Na_2CO_3. The eluant was acidified with 1 N HCl to pH 2, extracted with EA (30 ml × 3), and subsequently the solvent was removed under reduced pressure. The resulting crude residue was recrystallized from ethanol and dried to afford the target compound as white solid 1.98 g, yield: 46.5%. Mp 192–193 °C, IR (KBr, cm^{-1}): 3344 (NH), 3297 (OH), 1735 (O=C–OH), 1650 (O=C–NH), 1599 and 1514 (Ar), 1267 (C–N), 1161 and 1145 (C–O). ^1H NMR (DMSO-d_6, ppm): δ 12.16 (s, 1H, COOH), 10.04 (s, 1H, NH), 8.27 (d, 1H, NH, *J* = 7.92 Hz), 7.48 (d, 2H, Ar-H, *J* = 8.4 Hz), 7.37 (d, 1H, C=CH, *J* = 9.72 Hz), 7.16 (d, 1H, Ar-H, *J* = 4.32 Hz), 7.11 (m, 3H, Ar-H), 6.98 (d, 1H, Ar-H, *J* = 8.4 Hz), 6.68 (d, 1H, C=CH, *J* = 15.72 Hz), 4.57 (m, 1H, CH), 3.79 (s, 3H, $-OCH_3$), 3.78 (s, 3H, $-OCH_3$), 2.29 (m, 2H, CH_2), 2.08 (s, 3H, CH_3), 1.99 (m, 1H, CH_2), 1.88 (m, 1H, CH_2). ESI-MS: 427.6 [M+1].

The following L-*iso*-glutamine derivatives **A2–A13** were prepared according to the general procedure as described for the preparation of compound **A1**.

5.2.6. (*S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(4-fluorophenylamino)-5-oxopentanoic acid (A2)

Yield: 32.7%, mp 171.0–173.0 °C, IR (KBr, cm^{-1}): 3318 (NH), 3289 (OH), 1703 (O=C–OH), 1672 (O=C–NH), 1599 and 1510 (Ar), 1265 (C–N), 1159 and 1141 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.12 (s, 1H, COOH), 10.05 (s, 1H, NH), 8.26 (d, 1H, NH, J = 7.86 Hz), 7.62 (q, 2H, Ar-H), 7.38 (d, 1H, C=CH, J = 15.72 Hz), 7.14 (m, 4H, Ar-H), 6.98 (d, 1H, Ar-H, J = 8.34 Hz), 6.68 (d, 1H, C=CH, J = 15.78 Hz), 4.54 (m, 1H, CH), 3.80 (s, 3H, –OCH_3), 3.79 (s, 3H, –OCH_3), 2.31 (m, 2H, CH_2), 1.90 (m, 1H, CH_2), 1.82 (m, 1H, CH_2). ESI-MS: 431.7 [M+1].

5.2.7. (*S*,*E*)-5-(4-Chlorophenylamino)-4-(3-(3,4-dimethoxyphenyl)acrylamido)-5-oxopentanoic acid (A3)

Yield: 36.1%, mp 187.0–188.0 °C, IR (KBr, cm^{-1}): 3316 (NH), 3281 (OH), 1701 (O=C–OH), 1652 (O=C–NH), 1597 and 1514 (Ar), 1265 (C–N), 1160 and 1143 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.13 (s, 1H, COOH), 10.23 (s, 1H, NH), 8.27 (d, 1H, NH, J = 7.8 Hz), 7.64 (d, 2H, Ar-H, J = 8.88 Hz), 7.39 (m, 3H, Ar-H), 7.38 (d, 1H, C=CH, J = 9.72 Hz), 7.11 (m, 4H, Ar-H), 6.98 (d, 1H, Ar-H, J = 2.4 Hz), 6.68 (d, 1H, C=CH, J = 21.78 Hz), 4.54 (m, 1H, CH), 3.80 (s, 3H, –OCH_3), 3.79 (s, 3H, –OCH_3), 2.30 (m, 2H, CH_2), 2.08 (m, 1H, CH_2), 1.99 (m, 1H, CH_2). ESI-MS: 447.6 [M+1].

5.2.8. (*S*,*E*)-5-(4-Bromophenylamino)-4-(3-(3,4-dimethoxyphenyl)acrylamido)-5-oxopentanoic acid (A4)

Yield: 33.9%, mp 186.0–187.2 °C, IR (KBr, cm^{-1}): 3334 (NH), 3288 (OH), 1741 (O=C–OH), 1653 (O=C–NH), 1596 and 1514 (Ar), 1271 (C–N), 1163 and 1145 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.12 (s, 1H, COOH), 10.23 (s, 1H, NH), 8.27 (d, 1H, NH, J = 5.87 Hz), 7.58 (d, 2H, Ar-H, J = 8.82 Hz), 7.49 (d, 2H, Ar-H, J = 8.76 Hz), 7.37 (d, 1H, C=CH, J = 9.72 Hz), 7.16 (d, 1H, Ar-H, J = 1.14 Hz), 7.12 (d, 1H, Ar-H, J = 8.88 Hz), 6.99 (d, 1H, Ar-H, J = 8.34 Hz), 6.67 (d, 1H, C=CH, J = 15.72 Hz), 4.53 (m, 1H, CH), 3.80 (s, 3H, –OCH_3), 3.79 (s, 3H, –OCH_3), 2.30 (m, 2H, CH_2), 2.09 (m, 1H, CH_2), 2.01 (m, 1H, CH_2). ESI-MS: 491.5 [M+1].

5.2.9. (*S*,*E*)-5-(3,5-Difluorophenylamino)-4-(3-(3,4-dimethoxyphenyl)acrylamido)-5-oxopentanoic acid (A5)

Yield: 35.3%, mp 190.0–191.0 °C, IR (KBr, cm^{-1}): 3322 (NH), 3280 (OH), 1710 (O=C–OH), 1657 (O=C–NH), 1617 and 1514 (Ar), 1266 (C–N), 1161 and 1140 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.17 (s, 1H, COOH), 10.54 (s, 1H, NH), 8.36 (d, 1H, NH, J = 7.56 Hz), 7.38 (d, 1H, C=CH, J = 15.72 Hz), 7.35 (m, 2H, Ar-H), 7.16 (d, 1H, Ar-H, J = 1.8 Hz), 7.12 (q, 1H, Ar-H), 7.06 (d, 1H, Ar-H, J = 8.4 Hz), 6.99 (d, 1H, Ar-H, J = 1.8 Hz), 6.66 (d, 1H, C=CH, J = 15.78 Hz), 4.50 (m, 1H, CH), 3.80 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.32 (m, 2H, CH_2), 2.01 (m, 1H, CH_2), 1.90 (m, 1H, CH_2). ESI-MS: 449.6 [M+1].

5.2.10. (*S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(2-fluorophenylamino)-5-oxopentanoic acid (A6)

Yield: 30.5%, mp 168.0–169.0 °C, IR (KBr, cm^{-1}): 3420 (NH), 3337 (OH), 1748 (O=C–OH), 1651 (O=C–NH), 1594 and 1512 (Ar), 1264 (C–N), 1162 and 1141 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.18 (s, 1H, COOH), 9.89 (s, 1H, NH), 8.32 (d, 1H, NH, J = 7.92 Hz), 7.81 (m, 1H, Ar-H), 7.38 (d, 1H, C=CH, J = 15.72 Hz), 7.38 (d, 1H, Ar-H, J = 10.8 Hz), 7.25 (m, 1H, Ar-H), 7.18 (m, 1H, Ar-H), 7.13 (d, 1H, Ar-H, J = 1.62 Hz), 6.99 (d, 1H, Ar-H, J = 8.34 Hz), 6.67 (d, 1H, C=CH, J = 7.56 Hz), 4.67 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.34 (m, 2H, CH_2), 2.03 (m, 1H, CH_2), 1.90 (m, 1H, CH_2). ESI-MS: 431.7 [M+1].

5.2.11. (*S*,*E*)-5-(2-Chlorophenylamino)-4-(3-(3,4-dimethoxyphenyl)acrylamido)-5-oxopentanoic acid (A7)

Yield: 39.3%, mp 125.0–126.0 °C, IR (KBr, cm^{-1}): 3421 (NH), 3336 (OH), 1748 (O=C–OH), 1651 (O=C–NH), 1596 and 1512 (Ar), 1264 (C–N), 1162 and 1141 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.10 (s, 1H, COOH), 9.88 (s, 1H, NH), 8.68 (d, 1H, NH, J = 1.84 Hz), 7.76 (d, 1H, Ar-H, J = 7.92 Hz), 7.49 (d, 1H, Ar-H, J = 8.01 Hz), 7.39 (d, 1H, C=CH, J = 15.72 Hz), 7.32 (d, 1H, Ar-H, J = 7.35 Hz), 7.18 (m, 1H, Ar-H), 7.13 (d, 1H, Ar-H, J = 6.79 Hz), 6.99 (d, 1H, Ar-H, J = 8.36 Hz), 6.66 (d, 1H, C=CH, J = 15.74 Hz), 4.63 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.28 (m, 2H, CH_2), 2.03 (m, 1H, CH_2), 1.92 (m, 1H, CH_2). ESI-MS: 447.6 [M+1].

5.2.12. (*S*,*E*)-5-(3-Chlorophenylamino)-4-(3-(3,4-dimethoxyphenyl)acrylamido)-5-oxopentanoic acid (A8)

Yield: 37.6%, mp 163.0–164.0 °C, IR (KBr, cm^{-1}): 3316 (NH), 3281 (OH), 1701 (O=C–OH), 1652 (O=C–NH), 1597 and 1514 (Ar), 1265 (C–N), 1160 and 1143 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.19 (s, 1H, COOH), 10.36 (s, 1H, NH), 8.35 (d, 1H, NH, J = 1.38 Hz), 7.82 (d, 1H, Ar-H, J = 1.74 Hz), 7.43 (m, 3H, Ar-H), 7.39 (d, 1H, C=CH, J = 7.68 Hz), 7.12 (m, 3H, Ar-H), 6.99 (d, 1H, Ar-H, J = 8.34 Hz), 6.66 (d, 1H, C=CH, J = 15.72 Hz), 4.51 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.33 (m, 2H, CH_2), 1.97 (m, 1H, CH_2), 1.90 (m, 1H, CH_2). ESI-MS: 447.5 [M+1].

5.2.13. (*S*,*E*)-5-(Benzylamino)-4-(3-(3,4-dimethoxyphenyl)-acrylamido)-5-oxopentanoic acid (A9)

Yield: 43.1%, mp 180.0–181.0 °C, IR (KBr, cm^{-1}): 3302 (NH), 1708 (O=C–OH), 1666 (O=C–NH), 1599 and 1511 (Ar), 1264 (C–N), 1159 and 1142 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.08 (s, 1H, COOH), 8.48 (s, 1H, NH), 8.12 (d, 1H, NH, J = 8.04 Hz), 7.36 (d, 1H, C=CH, J = 15.78 Hz), 7.32 (t, 1H, Ar-H), 7.23 (q, 1H, Ar-H), 7.16 (d, 1H, Ar-H, J = 1.14 Hz), 7.11 (d, 1H, Ar-H, J = 8.28 Hz), 6.99 (d, 1H, Ar-H, J = 8.34 Hz), 6.66 (d, 1H, C=CH, J = 15.72 Hz), 4.43 (m, 1H, CH), 4.28 (d, 2H, CH_2), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.26 (m, 2H, CH_2), 1.98 (m, 1H, CH_2), 1.82 (m, 1H, CH_2). ESI-MS: 427.6 [M+1].

5.2.14. (*S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(methylamino)-5-oxopentanoic acid (A10)

Yield: 53.1%, mp 108.0–109.0 °C, IR (KBr, cm^{-1}): 3300 (NH), 1707 (O=C–OH), 1668 (O=C–NH), 1606 and 1513 (Ar), 1261 (C–N), 1155 and 1139 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.10 (s, 1H, COOH), 8.09 (s, 1H, NH), 7.99 (d, 1H, NH, J = 1.78 Hz), 7.35 (d, 1H, C=CH, J = 8.4 Hz), 7.17 (d, 1H, Ar-H, J = 6.45 Hz), 7.16 (d, 1H, Ar-H, J = 7.92 Hz), 6.79 (d, 1H, Ar-H, J = 15.72 Hz), 6.84 (d, 1H, C=CH, J = 8.24 Hz), 4.53 (m, 1H, CH), 3.77 (s, 3H, –OCH_3), 3.73 (s, 3H, –OCH_3), 2.23 (m, 2H, CH_2), 2.06 (m, 1H, CH_2), 1.71 (s, 3H, CH_3). ESI-MS: 351.5 [M+1].

5.2.15. (*S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(ethylamino)-5-oxopentanoic acid (A11)

Yield: 40.7%, mp 112.0–113.0 °C, IR (KBr, cm^{-1}): 3298 (NH), 1706 (O=C–OH), 1661 (O=C–NH), 1597 and 1510 (Ar), 1259 (C–N), 1156 and 1137 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.11 (s, 1H, COOH), 8.09 (s, 1H, NH), 7.98 (d, 1H, NH, J = 7.56 Hz), 7.56 (d, 1H, C=CH, J = 8.01 Hz), 6.97 (d, 1H, Ar-H, J = 7.2 Hz), 6.78 (d, 1H, Ar-H, J = 1.8 Hz), 6.67 (d, 1H, Ar-H, J = 9.2 Hz), 6.61 (d, 1H, C=CH, J = 15.78 Hz), 4.53 (m, 1H, CH), 3.75 (s, 3H, –OCH_3), 3.73 (s, 3H, –OCH_3), 3.24 (m, 2H, CH_2), 2.23 (m, 2H, CH_2), 2.05 (m, 1H, CH_2), 1.28 (m, 3H, CH_3). ESI-MS: 365.6 [M+1].

5.2.16. (*S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-oxo-5-(propylamino)pentanoic acid (A12)

Yield: 37.0%, mp 126.0–127.0 °C, IR (KBr, cm^{-1}): 3282 (NH), 1708 (O=C–OH), 1643 (O=C–NH), 1611 and 1512 (Ar), 1262 (C–

N), 1160 and 1141 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.09 (s, 1H, COOH), 8.11 (s, 1H, NH), 7.99 (d, 1H, NH, J = 5.7 Hz), 7.35 (d, 1H, C=CH, J = 15.72 Hz), 7.16 (d, 1H, Ar-H, J = 1.26 Hz), 7.11 (d, 1H, Ar-H, J = 8.28 Hz), 6.98 (d, 1H, Ar-H, J = 8.34 Hz), 6.66 (d, 1H, C=CH, J = 15.06 Hz), 4.35 (m, 1H, CH), 3.78 (s, 3H, –OCH_3), 3.72 (s, 3H, –OCH_3), 3.00 (m, 2H, CH_2), 2.22 (m, 2H, CH_2), 1.93 (m, 1H, CH_2), 1.87 (m, 1H, CH_2), 1.39 (m, 2H, CH_2), 0.83 (m, 3H, CH_3). ESI-MS: 379.7 [M+1].

5.2.17. (*S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(isopropylamino)-5-oxopentanoic acid (A13)

Yield: 38.8%, mp 157.0–158.0 °C, IR (KBr, cm^{-1}): 3373 (NH), 3327 (OH), 1737 (O=C–OH), 1642 (O=C–NH), 1609 and 1516 (Ar), 1262 (C–N), 1160 and 1136 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.13 (s, 1H, COOH), 8.05 (s, 1H, NH), 7.90 (d, 1H, NH, J = 7.68 Hz), 7.35 (d, 1H, C=CH, J = 15.72 Hz), 7.16 (m, 1H, Ar-H), 7.10 (d, 1H, Ar-H, J = 1.32 Hz), 6.98 (d, 1H, Ar-H, J = 8.34 Hz), 6.68 (d, 1H, C=CH, J = 15.72 Hz), 4.36 (m, 1H, CH), 4.03 (m, 1H, CH), 3.82 (s, 3H, –OCH_3), 3.79 (s, 3H, –OCH_3), 2.22 (m, 2H, CH_2), 1.88 (m, 1H, CH_2), 1.77 (m, 1H, CH_2). ESI-MS: 379.7 [M+1].

5.2.18. (*S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(2-methoxy-2-oxoethylamino)-5-oxopentanoic acid (A14)

Preparation of L-Amino acid methyl ester hydrochloride: To a suspension of glycine (15.0 g, 200 mmol) in anhydrous methanol (150 ml) at 0 °C was pumped slowly in dried HCl gas until solution was completely clear. Cool in a refrigerator, preferably overnight. Remove the solvent and small amount methanol was added again to remove the excess HCl. The collected white precipitate was recrystallized from methanol/diethyl ether (1:4) to give methyl 2-aminoacetate hydrochloride (20 g, 80%). Mp 175–176 °C, IR (KBr, cm^{-1}): 3215.0–3523.0 (NH), 2935.2 (CH), 1676.3 (C=O), 1129.3 (C–O).

To a solution of compound **5** (3.5 g, 10 mmol) in $CHCl_3$ (20 ml) at −5 °C was added a solution of DCC (2.06 g, 10 mmol) in 50 ml $CHCl_3$ in small portions. After completion the DCC, a solution of methyl 2-aminoacetate hydrochloride (10 mmol) in 20 ml $CHCl_3$ was added dropwise. Then the mixture was stirred at this temperature for 5 h. Filtered off the DCU and washed with dilute Na_2CO_3 solution. The eluant was acidified with 1 N HCl to pH 2, extracted with EA (30 ml × 3), and subsequently the solvent was removed under reduced pressure. The resulting crude residue was recrystallized from ethanol and dried to afford the target compound as white solid, yield: 46.1%. Mp 172.0–173.0 °C, IR (KBr, cm^{-1}): 3326 (NH), 3286 (OH), 1754 (O=C–OH), 1651 (O=C–NH), 1597 and 1513 (Ar), 1264 (C–N), 1160 and 1140 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.06 (s, 1H, COOH), 8.40 (s, 1H, NH), 8.13 (d, 1H, NH, J = 8.1 Hz), 7.36 (d, 1H, C=CH, J = 15.72 Hz), 7.16 (s, 1H, Ar-H), 7.11 (q, 1H, Ar-H), 6.99 (d, 1H, Ar-H, J = 8.34 Hz), 6.66 (d, 1H, C=CH, J = 15.72 Hz), 4.46 (m, 1H, CH), 3.80 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 3.63 (s, 3H, –OCH_3), 3.60 (s, 3H, –OCH_3), 2.28 (m, 2H, CH_2), 1.96 (m, 1H, CH_2), 1.81 (m, 1H, CH_2). ESI-MS: 409.6 [M+1].

5.2.19. (*4S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(1-methoxy-1-oxopropan-2-ylamino)-5-oxopentanoic acid (A15)

Yield: 38.8%, mp 163.0–164.0 °C, IR (KBr, cm^{-1}): 3326 (NH), 3300 (OH), 1755 (O=C–OH), 1650 (O=C–NH), 1590 and 1518 (Ar), 1244 (C–N), 1164 and 1141 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.21 (s, 1H, COOH), 8.46 (s, 1H, NH), 8.11 (d, 1H, NH, J = 8.20 Hz), 7.37 (d, 1H, C=CH, J = 15.71 Hz), 7.16 (d, 1H, Ar-H, J = 1.43 Hz), 7.11 (q, 1H, Ar-H), 6.97 (d, 1H, Ar-H, J = 8.39 Hz), 6.68 (d, 1H, C=CH, J = 15.75 Hz), 4.49 (m, 1H, CH), 3.80 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 3.62 (s, 3H, –OCH_3), 2.30 (m, 2H, CH_2), 1.98 (m, 1H, CH_2), 1.83 (m, 1H, CH_2), 1.30 (s, 3H, CH_3). ESI-MS: 423.5 [M+1].

5.2.20. (*4S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(1-methoxy-3-methyl-1-oxobutan-2-ylamino)-5-oxopentanoic acid (A16)

Yield: 39.5%, mp 143.1–144.5 °C, IR (KBr, cm^{-1}): 3326 (NH), 3284 (OH), 1740 (O=C–OH), 1651 (O=C–NH), 1616 and 1514 (Ar), 1262 (C–N), 1169 and 1140 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.00 (s, 1H, COOH), 8.22 (s, 1H, NH), 8.13 (d, 1H, NH, J = 8.03 Hz), 7.36 (d, 1H, C=CH, J = 15.71 Hz), 7.16 (d, 1H, Ar-H, J = 1.41 Hz), 7.11 (q, 1H, Ar-H), 6.98 (d, 1H, Ar-H, J = 8.37 Hz), 6.68 (d, 1H, C=CH, J = 15.73 Hz), 4.18 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 3.61 (s, 3H, –OCH_3), 2.50 (m, 2H, CH_2), 2.08 (m, 1H, CH), 1.93 (m, 1H, CH_2), 1.81 (m, 1H, CH_2), 0.90 (s, 3H, CH_3), 0.88 (s, 3H, CH_3). ESI-MS: 451.3 [M+1].

5.2.21. (*4S*,*E*)-5-(1,4-Dimethoxy-1,4-dioxobutan-2-ylamino)-4-(3-(3,4-dimethoxyphenyl)acrylamido)-5-oxopentanoic acid (A17)

Yield: 41.2%, mp 155.0–156.0 °C, IR (KBr, cm^{-1}): 3290 (NH), 3284 (OH), 1731 (O=C–OH), 1644 (O=C–NH), 1613 and 1512 (Ar), 1263 (C–N), 1165 and 1142 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.07 (s, 1H, COOH), 8.52 (s, 1H, NH), 8.12 (d, 1H, NH, J = 8.19 Hz), 7.39 (d, 1H, C=CH, J = 2.06 Hz), 7.16 (s, 1H, Ar-H), 7.11 (d, 1H, Ar-H, J = 8.31 Hz), 6.97 (d, 1H, Ar-H, J = 8.39 Hz), 6.69 (d, 1H, C=CH, J = 4.25 Hz), 4.68 (m, 1H, CH), 4.47 (m, 1H, CH), 3.78 (s, 3H, –OCH_3), 3.76 (s, 3H, –OCH_3), 3.64 (s, 3H, OCH_3), 3.60 (s, 3H, –OCH_3), 2.86 (m, 1H, CH), 2.76 (m, 1H, CH), 2.28 (m, 2H, CH_2), 1.90 (m, 1H, CH_2), 1.80 (m, 1H, CH_2). ESI-MS: 481.4 [M+1].

5.2.22. (*4S*,*E*)-4-(3-(3,4-Dimethoxyphenyl)acrylamido)-5-(3-(4-fluorophenyl)-1-methoxy-1-oxopropan-2-ylamino)-5-oxopentanoic acid (A18)

Yield: 35.2%, mp 116.0–117.0 °C, IR (KBr, cm^{-1}): 3149 (NH), 3263 (OH), 1721 (O=C–OH), 1750 (O=C–NH), 1616 and 1512 (Ar), 1264 (C–N), 1160 and 1140 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 12.01 (s, 1H, COOH), 8.27 (s, 1H, NH), 8.01 (d, 1H, NH, J = 8.4 Hz), 7.36 (d, 1H, C=CH, J = 15.66 Hz), 7.25 (m, 2H, Ar-H), 7.16 (d, 1H, Ar-H, J = 1.86 Hz), 7.10 (m, 1H, Ar-H), 7.02 (m, 2H, Ar-H), 6.99 (d, 1H, Ar-H, J = 8.4 Hz), 6.65 (d, 1H, C=CH, J = 15.72 Hz), 4.45 (m, 1H, CH), 4.43 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 3.06 (m, 1H, CH_2), 2.86 (m, 1H, CH_2), 2.03 (m, 2H, CH_2), 1.76 (m, 1H, CH_2), 1.63 (m, 1H, CH_2). ESI-MS: 503.3 [M+1].

5.2.23. (*S*,*E*)-2-(3-(3,4-Dimethoxyphenyl)acrylamido)-N^1,N^5-dip-tolylpentanediamide (B1)

To a solution of compound **5** (3.5 g, 10 mmol) in $CHCl_3$ (20 ml) at −5 °C was added a solution of DCC (4.12 g, 2 equiv, 20 mmol) in 100 ml $CHCl_3$ in small portions. After completion the DCC, a solution of *p*-toluidine (2.16 g, 2.05 eq, 21 mmol) in 30 ml $CHCl_3$ was added dropwise. Then the mixture was stirred at this temperature until the solid has almost completely dissolved (about 5 h). The insoluble material DCU was filtered off and washed with cold 1 N Na_2CO_3. The eluant was purified by flash column chromatography with cyclohexane–EA (4:1 to 1:1) to provide 1.68 g of white solid, yield 32.6%, mp 240.0–241.0 °C, IR (KBr, cm^{-1}): 3273 (NH), 1652 (O=C–NH), 1600 and 1514 (Ar), 1260 (C–N), 1160 and 1138 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 10.08 (s, 1H, NH), 9.85 (s, 1H, NH), 8.29 (d, 1H, NH, J = 8.04 Hz), 7.51 (d, 2H, Ar-H, J = 8.4 Hz), 7.44 (d, 2H, Ar-H, J = 8.4 Hz), 7.38 (d, 1H, C=CH, J = 15.72 Hz), 7.16 (d, 1H, Ar-H, J = 7.86 Hz), 7.11 (d, 3H, Ar-H, J = 8.4 Hz), 7.06 (d, 2H, Ar-H, J = 8.4 Hz), 6.98 (d, 1H, Ar-H, J = 8.4 Hz), 6.71 (d, 1H, C=CH, J = 15.72 Hz), 4.57 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.38 (m, 2H, CH_2), 2.25 (s, 3H, CH_3), 2.24 (s, 3H, CH_3), 2.07 (m, 1H, CH_2), 1.96 (m, 1H, CH_2). ESI-MS: 516.8 [M+1].

The other L-*iso*-glutamine derivatives **B2–B7** were prepared according to the general procedure as described for the preparation of compound **B1**.

5.2.24. (*S*,*E*)-2-(3-(3,4-Dimethoxyphenyl)acrylamido)-N^1,N^5-bis(4-fluorophenyl)pentanediamide (B2)

Yield: 30.0%, mp 188.0–189.0 °C, IR (KBr, cm^{-1}): 3277 (NH), 1659 (O=C–NH), 1615 & 1509 (Ar), 1261 (C–N), 1161 and 1145 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 10.25 (s, 1H, NH), 10.00 (s, 1H, NH), 8.34 (d, 1H, NH, J = 6.12 Hz), 7.65 (q, 2H, Ar-H), 7.58 (q, 2H, Ar-H), 7.38 (d, 1H, C=CH, J = 15.72 Hz), 7.16 (t, 3H, Ar-H), 7.11 (t, 3H, Ar-H), 6.98 (d, 1H, Ar-H, J = 2.34 Hz), 6.70 (d, 1H, C=CH, J = 9.72 Hz), 4.57 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.45 (m, 2H, CH_2), 2.09 (m, 1H, CH_2), 1.96 (m, 1H, CH_2). ESI-MS: 524.7 [M+1].

5.2.25. (*S*,*E*)-N^1,N^5-Bis(4-chlorophenyl)-2-(3-(3,4-dimethoxyphenyl)acrylamido)pentanediamide (B3)

Yield: 38.3%, mp 206.0–207.0 °C, IR (KBr, cm^{-1}): 3271 (NH), 1652 (O=C–NH), 1596 and 1514 (Ar), 1261 (C–N), 1160 and 1138 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 10.28 (s, 1H, NH), 10.04 (s, 1H, NH), 8.30 (d, 1H, NH, J = 7.86 Hz), 7.66 (d, 2H, Ar-H, J = 8.22 Hz), 7.59 (d, 2H, Ar-H, J = 2.82 Hz), 7.38 (m, 3H, Ar-H), 7.32 (d, 1H, C=CH, J = 8.88 Hz), 7.15 (d, 1H, Ar-H, J = 1.62 Hz), 7.11 (d, 1H, Ar-H, J = 1.62 Hz), 6.99 (d, 1H, Ar-H, J = 8.34 Hz), 6.68 (d, 1H, C=CH, J = 15.78 Hz), 4.58 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.45 (m, 2H, CH_2), 2.09 (m, 1H, CH_2), 1.97 (m, 1H, CH_2). ESI-MS: 556.5 [M+1].

5.2.26. (*S*,*E*)-N^1,N^5-Bis(4-bromophenyl)-2-(3-(3,4-dimethoxyphenyl)acrylamido)pentanediamide (B4)

Yield: 37.7%, mp 210.0–212.0 °C, IR (KBr, cm^{-1}): 3268 (NH), 1648 (O=C–NH), 1591 and 1515 (Ar), 1262 (C–N), 1160 and 1138 (C-O). ^{1}H NMR^{1}H NMR (DMSO-d_6, ppm): δ 10.28 (s, 1H, NH), 10.04 (s, 1H, NH), 8.30 (d, 1H, NH, J = 7.86 Hz), 7.60 (q, 2H, Ar-H), 7.54 (d, 2H, Ar-H, J = 8.88 Hz), 7.50 (d, 2H, Ar-H, J = 8.88 Hz), 7.44 (d, 2H, Ar-H, J = 7.02 Hz), 7.38 (d, 1H, C=CH, J = 15.78 Hz), 7.16 (d, 1H, Ar-H, J = 1.8 Hz), 7.11 (q, 1H, Ar-H), 6.99 (d, 1H, Ar-H, J = 8.34 Hz), 6.68 (d, 1H, C=CH, J = 15.72 Hz), 4.58 (m, 1H, CH), 3.80 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.43 (m, 2H, CH_2), 2.09 (m, 1H, CH_2), 1.97 (m, 1H, CH_2). ESI-MS: 644.3 [M+1].

5.2.27. (*S*,*E*)-2-(3-(3,4-Dimethoxyphenyl)acrylamido)-N^1,N^5-bis(2-fluorophenyl)pentanediamide (B5)

Yield: 38.6%, mp 187.0–188.0 °C, IR (KBr, cm^{-1}): 3326 (NH), 1626 (O=C–NH), 1575 and 1515 (Ar), 1261 (C–N), 1159 and 1139 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 9.87 (s, 1H, NH), 9.72 (s, 1H, NH), 8.31 (d, 1H, NH, J = 7.92 Hz), 7.76 (m, 2H, Ar-H), 7.41 (d, 1H, C=CH, J = 15.72 Hz), 7.23 (m, 2H, Ar-H), 7.15 (m, 3H, Ar-H), 7.15 (m, 3H, Ar-H), 7.10 (m, 3H, Ar-H), 6.99 (d, 1H, Ar-H, J = 8.34 Hz), 6.70 (d, 1H, C=CH, J = 15.72 Hz), 4.72 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.53 (m, 2H, CH_2), 2.13 (m, 1H, CH_2), 1.98 (m, 1H, CH_2). ESI-MS: 524.5 [M+1].

5.2.28. (*S*,*E*)-N^1,N^5-Bis(3,5-difluorophenyl)-2-(3-(3,4-dimethoxyphenyl)acrylamido)pentanediamide (B6)

Yield: 44.6%, mp 220.0–222.0 °C, IR (KBr, cm^{-1}): 3281 (NH), 1670 (O=C–NH), 1612 and 1516 (Ar), 1260 (C–N), 1166 and 1138 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 10.52 (s, 1H, NH), 10.27 (s, 1H, NH), 8.36 (d, 1H, NH, J = 7.74 Hz), 7.38 (d, 1H, C=CH, J = 15.78 Hz), 7.36 (m, 2H, Ar-H), 7.27 (q, 2H, Ar-H), 7.15 (d, 1H, Ar-H, J = 1.8 Hz), 7.10 (q, 1H, Ar-H), 6.98 (d, 1H, Ar-H, J = 8.4 Hz), 6.92 (m, 1H, Ar-H), 6.83 (m, 1H, Ar-H), 6.67 (d, 1H, C=CH, J = 15.72 Hz), 4.56 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 2.47 (m, 2H, CH_2), 2.12 (m, 1H, CH_2), 1.98 (m, 1H, CH_2). ESI-MS: 560.5 [M+1].

5.2.29. (*S*,*E*)-2-[3-(3,4-Dimethoxyphenyl)acrylamido]-N^1,N^5-[(*R*)-1,4-dimethoxy-1,4-dioxobutan]pentanediamide (B7)

Yield: 42.3%, mp 179.0–181.0 °C, IR (KBr, cm^{-1}): 3291 (NH), 1645 (O=C–NH), 1613 and 1514 (Ar), 1264 (C–N), 1161 and 1140 (C–O). ^{1}H NMR (DMSO-d_6, ppm): δ 8.47 (d, 1H, NH, J = 7.91 Hz), 8.32 (d, 1H, NH, J = 7.88 Hz), 8.07 (d, 1H, NH, J = 8.21 Hz), 7.36 (d, 1H, C=CH, J = 15.71 Hz), 7.16 (d, 1H, Ar-H, J = 1.72 Hz), 7.10 (d, 1H, Ar-H, J = 6.58 Hz), 6.98 (d, 1H, Ar-H, J = 8.35 Hz), 6.67 (d, 1H, C=CH, J = 15.85 Hz), 4.62 (m, 1H, CH), 4.32 (m, 1H, CH), 3.79 (s, 3H, –OCH_3), 3.78 (s, 3H, –OCH_3), 3.63 (s, 3H, –OCH_3), 3.58 (s, 3H, –OCH_3), 2.82 (m, 2H, CH_2), 2.76 (m, 2H, CH_2), 2.18 (m, 2H, CH_2), 1.93 (m, 1H, CH_2), 1.73 (m, 1H, CH_2). ESI-MS: 624.6 [M+1].

6. Enzymatic inhibiton assay (in vitro)

6.1. In vitro APN assay

The IC_{50} values against APN were determined using L-Leu-*p*-nitroanilide as substrate and microsomal aminopeptidase from Porcine Kidney Mocrosomes (Sigma) as the enzyme in 50 mM PBS, pH 7.2 at 37 °C.[31] The hydrolysis of the substrate was monitored by following the change in the absorbance measured at 405 nm with the UV–vis spectrophotometer Pharmacia LKB, Biochrom 4060. All solutions of inhibitors were prepared in the assay buffer, and pH was adjusted to 7.5 by the addition of 0.1 M HCl or 0.1 M NaOH. All inhibitors were preincubated with APN for 30 min at room temperature. The assay mixture, which contained the inhibitor solution (concentration dependent on the inhibitor), the enzyme solution (4 μg/ml final concentration), and the assay buffer, was adjusted to 200 μL.

6.2. In vitro MMP-2 assay

MMP-2, also called as Gelatinase A, assay was performed as described by Baragi et al.[32] The gelatinase, substrate and inhibitor were dissolved in sodium borate (pH 8.5, 50 mmol/L) and incubated for 30 min at 37 °C, and then 0.03% trinitrobenzenesulfonic acid (TNBS, Sigma) was added and incubated for another 20 min, the resulting solution was detected under 450 nm wavelength to gain absorption.

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References and notes

1. Fukasawa, K.; Fujii, H.; Saitoh, Y.; Koizumi, K.; Aozuka, Y.; Sekine, K.; Yamada, M.; Saiki, I.; Nishikawa, K. *Cancer Lett.* **2006**, *243*, 135.
2. Riemann, D.; Kehlen, A.; Langner, J. *Immunol. Today* **1999**, *20*, 83.
3. Fujii, H.; Nakajima, M.; Saiki, I.; Yoneda, J.; Azuma, I.; Tsuruo, T. *Clin. Exp. Metastasis* **1995**, *13*, 337.
4. Ishii, K.; Usui, S.; Sugimura, Y.; Toshida, S.; Hioki, T.; Tatematsu, M.; Yamamoto, H.; Hirano, K. *Int. J. Cancer* **2001**, *92*, 49.
5. Tomanek, R. J.; Schatteman, G. C. *Anat. Rec.* **2000**, *261*, 126.
6. Bhagwat, S. V.; Lahdenranta, J.; Giordano, R.; Arap, W.; Pasqualini, R.; Shapiro, L. H. *Blood* **2001**, *97*, 652.
7. Hashida, H.; Takabayashi, A.; Kanai, M.; Adachi, M.; Kondo, K.; Kohno, N.; Yamaoka, Y.; Miyake, M. *Gastroenterology* **2002**, *122*, 376.
8. Mina-Osorio, P. *Trends Mol. Med.* **2008**, *14*, 361.

9. Xu, W. F.; Li, Q. B. *Curr. Med. Chem. Anti-cancer Agents* **2005**, *5*, 281.
10. Babine, R. E.; Bender, S. L. *Chem. Rev.* **1997**, *97*, 1359.
11. Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305.
12. Umezawa, H.; Aoyagi, T.; Suda, H.; Hamada, M.; Takeuchi, T. *J. Antibiot. (Tokyo)* **1976**, *29*, 97.
13. Harbut, M. B.; Velmourougane, G.; Reiss, G.; Chandramohanadas, R.; Greenbaum, D. C. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5932.
14. Grzywa, R.; Oleksyszyn, J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3734.
15. Ito, K.; Nakajima, Y.; Onohara, Y.; Takeo, M.; Nakashima, K.; Matsubara, F.; Ito, T.; Yoshimoto, T. *J. Biol. Chem.* **2006**, *281*, 33664.
16. Addlagatta, A.; Gay, L.; Matthews, B. W. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 13339.
17. Tu, G.; Li, S.; Huang, H.; Li, G.; Xiong, F.; Mai, X.; Zhu, H.; Kuang, B.; Xu, W. F. *Bioorg. Med. Chem.* **2008**, *16*, 6663.
18. Wang, Q.; Chen, M.; Zhu, H.; Zhang, J.; Fang, H.; Wang, B.; Xu, W. *Bioorg. Med. Chem.* **2008**, *16*, 5473.
19. Shang, L.; Wang, Q.; Fang, H.; Mu, J.; Wang, X.; Yuan, Y.; Wang, B.; Xu, W. *Bioorg. Med. Chem.* **2008**, *16*, 9984.
20. Li, Q.; Fang, H.; Xu, W. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2935.
21. (a) Burzynski, S. R. *Drugs Exp. Clin. Res* **1986**, *12*, 1; (b) Khalid, M.; Burzynski, S. R. *Drugs Exp. Clin. Res.* **1987**, *13*, 57.
22. Burzynski, S. R.; Weaver, R. A.; Janicki, T.; Szymkowski, B.; Jurida, G.; Khan, M.; Dolgopolov, V. *Integr. Cancer. Ther.* **2005**, *4*, 168.
23. Badria, F.; Mabed, M.; El-Awadi, M.; Abou-Zeid, L.; Al-Nashar, E.; Hawas, S. *Cancer Lett.* **2000**, *157*, 57.
24. Kumabe, T.; Tsuda, H.; Uchida, M.; Ogoh, Y.; Hayabuchi, N.; Sata, M.; Nakashima, O.; Hara, H. *Oncol. Rep.* **1998**, *5*, 1363.
25. Kawabata, K.; Yamamoto, T.; Hara, A.; Shimizu, M.; Yamada, Y.; Matsunaga, K.; Tanaka, T.; Mori, H. *Cancer Lett.* **2000**, *157*, 15.
26. Cione, E.; Tucci, P.; Senatore, V.; Perri, M.; Trombino, S.; Iemma, F.; Picci, N.; Genchi, G. *J. Bioenerg. Biomembr.* **2008**, *40*, 19.
27. Shim, J. S.; Kim, J. H.; Cho, H. Y.; Yum, Y. N.; Kim, S. H.; Park, H. J.; Shim, B. S.; Choi, S. H.; Kwon, H. J. *Chem. Biol.* **2003**, *10*, 695.
28. Nishida, Y.; Miyamori, H.; Thompson, E. W.; Takino, T.; Endo, Y.; Sato, H. *Cancer Res.* **2008**, *68*, 9096.
29. Bjorklund, M.; Koivunen, E. *Biochim. Biophys. Acta* **2005**, *1755*, 37.
30. SYBYL6.91, Tripos Associates. 1699 S. Hanley Road, Suite 303. St. Louis, MO 63144-2913, 2003.
31. Lejczak, B.; Kafarski, P.; Zygmunt, J. *Biochemistry* **1989**, *28*, 3549.
32. Baragi, V. M.; Shaw, B. J.; Renkiewicz, R. R.; Kuipers, P. J.; Welgus, H. G.; Mathrubutham, M.; Cohen, J. R.; Rao, S. K. *Matrix Biol.* **2000**, *19*, 267.