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Design, synthesis and biological evaluation of novel amino acid ureido derivatives as aminopeptidase N/CD13 inhibitors

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ABSTRACT

A series of amino acid ureido derivatives as aminopeptidase N (APN/CD13) inhibitors were synthesized and evaluated for their APN inhibitory activities and anti-cancer effects. The results showed that most of these amino acid ureido derivatives exhibited good inhibition against APN, several of which were better than Bestatin. The most active compound **12j** (IC_{50} = 1.1 μM, compared with Bestatin IC_{50} = 8.1 μM) not only possessed much better APN inhibitory activity and anti-proliferation effect on cancer cells, but also exhibited significant block effect of human cancer cell invasion compared with the positive control, Bestatin. These amino acid ureido derivatives could be possibly developed as new APN inhibitors for cancer chemotherapy in the future.

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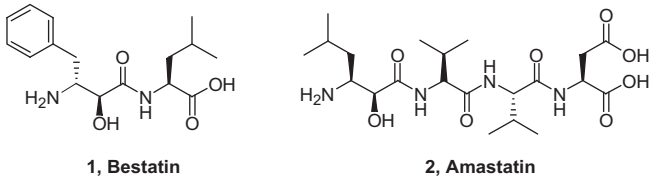
1. Introduction

Aminopeptidase N is a zinc-dependent integral transmembrane peptidase, belonging to M1 family,[1] which is distributed in various tissues and cell types.[2] As an exopeptidase, APN is reported to be involved in the cleavage of neutral amino acids from the N-terminals of biological oligopeptides, including enkephalins, neurokinins,[3] angiotensins and some cytokines.[4] APN is tightly related to various human cancer growths, and considered to be a cancer chemotherapeutic target for long time. Recently, a high level of its neutral cell surface exopeptidase activity to the degradation of extra cellular matrix (ECM)[5] has been detected on various highly metastatic human malignant tumor cells lines,[6] including melanoma, prostate, ovarian, colon, renal and pancreas carcinomas, as well as in some leukemias and lymphomas,[7] indicating that APN is not only responsible in the progression of cancer proliferation and cancer induced angiogenesis,[8] but also in the process of cancer invasion and metastasis.[9] Recently, anti-metastasis agents become increasingly attractive as promising agents in cancer treatment, and several APN inhibitors have been studied for years. Bestatin (**1**, Fig. 1) as an immuno-regulator on the market for leukemia chemotherapy, was reported to display significant block effects on the invasion of human malignant cancer cells in vitro[9] at higher concentration.[10] Another reported APN inhibitor, Amastatin (**2**, Fig. 1), also showed anti-invasion effect by blocking APN activity.[11] Recent reports suggest that APN inhibitors could also have the potential to be cancer chemotherapeutic agents according to their potent anti-proliferation and anti-invasion effects.[12]

In our previous studies, we designed and synthesized a series of L-lysine ureido derivatives[13] exhibiting potent APN inhibitory effects, several of which were similar to Bestatin. In this study, we kept the hydroxamate as the zinc-binding group (ZBG),[14] and varied the L-lysine side with other α-amino acids (R_1) and the R_2 substitutes, to make a preliminary exploration about their influence of the APN inhibitory activity. The ureido group as the ideal bioisostere of amide was still kept as the linker between R_1 and the R_2 substitutes, which would be helpful for keeping the conformation and improving the stability to digestive enzymes. Carbamate was also introduced in some cases as the counterpart of the ureido linker (Fig. 2).

2. Chemistry

The synthetic method of **4a–4i** is shown in Scheme 1. The ureido or carbamate linker were synthesized from the isocyanates of



1, Bestatin 2, Amastatin

Figure 1. Structures of reported APN inhibitors: Bestatin and Amastatin.

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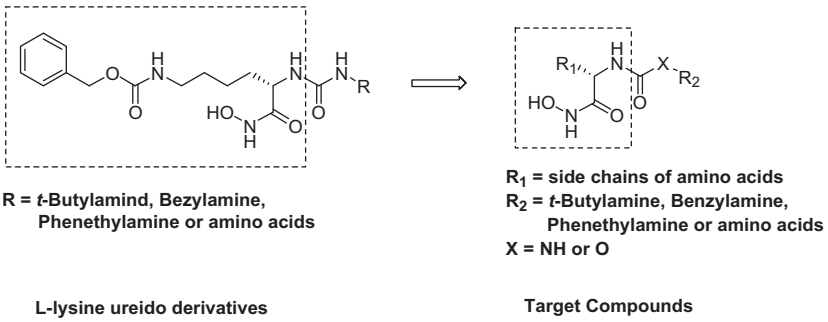
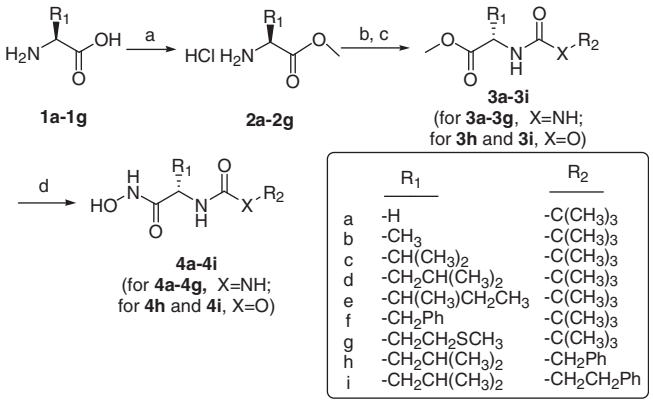
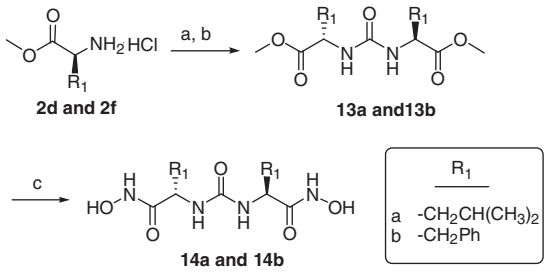


Figure 2. Target compounds.



Scheme 1. Reagents and conditions: (a) Acetyl chloride, MeOH, reflux; (b) triphosgene, $NaHCO_3$, DCM, ice-bath; (c) *t*-butylamine, BzOH, of $PhCH_2CH_2OH$, TEA and DCM, room temperature; (d) NH_2OK, MeOH, 4–6 h.



Scheme 3. Reagents and conditions: (a) Triphosgene, $NaHCO_3$, DCM, ice-bath; (b) methyl ester hydrochlorides of L-Leu of L-Phe, TEA and DCM, room temp, 1–2 h; (c) NH_2OK, MeOH, 4–6 h.

the chosen amino acids,[15] then coupled with the corresponding amino acids, amines or alcohols. Without further purification, they were directly transformed into hydroxamic acids as the target products. The main synthetic methods of the amino acid ureido derivatives **12a–12m** are shown in Scheme 2. With benzylamine, phenethylamine or cyclohexylamine as the start materials, they were converted into isocyanates, and then coupled with amino acid methyl esters, to obtain the ureido linker. Finally the methyl ester was transformed into hydroxamic acids as the target compounds. Ureido derivatives of double amino acids **14a** and **14b** were synthesized according to Scheme 3.

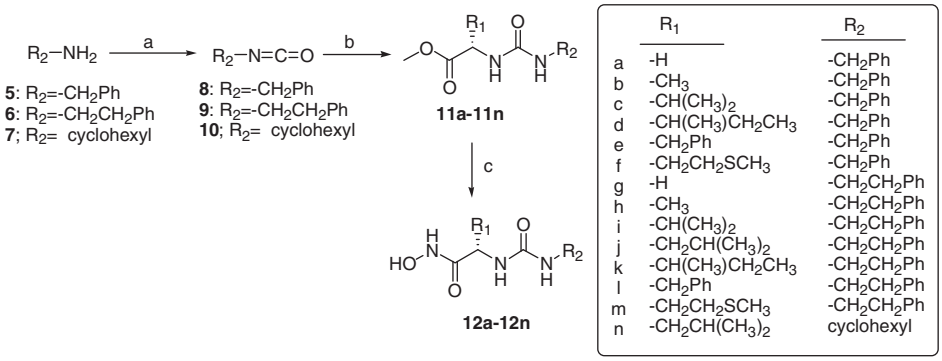
3. Result and discussion

In this paper, besides anti-proliferation, we also investigated the anti-invasion effects of APN inhibitors on cancer cells. Therefore, the synthesized amino acid ureido derivatives were assayed for their enzyme inhibitory activity using two types of APN from porcine kidney (Microsomal, Sigma) and ES-2 human ovarian cancer cell surface respectively. The inhibitors' selectivity over MMP-2 (Recombinant, Sigma) was also assayed. In the anti-invasion assay, we only chose the most potent compounds to be tested for their effects on ES-2 cell proliferation, migration, and invasion, to be compared with Bestatin as the positive control.

3.1. Enzyme inhibition assay

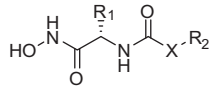
3.1.1. Enzyme inhibitory activity of the amino acid ureido derivatives towards APN from porcine kidney (Microsomal, Sigma)

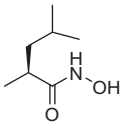
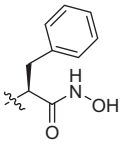
The enzyme inhibitory activities of all the target compounds in Table 1 were tested as a preliminary screening. The potencies of the compounds varied depending on the substitutes. Benzyl and phenethyl groups as R_2 showed to be very beneficial for the ureido derivatives to be potent APN inhibitors (**12a**–**12m**), as well as for the two carbamate derivatives (**4h** and **4i**). And we can see that



Scheme 2. Reagents and conditions: (a) Triphosgene, toluene, reflux; (b) methyl ester hydrochlorides of L-Gly, L-Ala, L-Val, L-Leu, L-IIe, L-Phe, or L-Met, TEA and DCM, room temp, 1–2 h; (c) NH_2OK, MeOH, 4–6 h.

Table 1
The structures and IC_{50} values of the target compounds towards APN from porcine kidney (Microsomal, Sigma)



Compounds	R_1	R_2	X	IC_{50} (μM) towards APN[a]
4a	–H	$-C(CH_3)_3$	NH	>100
4b	$-CH_3$	$-C(CH_3)_3$	NH	>100
4c	$-CH(CH_3)_2$	$-C(CH_3)_3$	NH	>100
4d	$-CH_2CH(CH_3)_2$	$-C(CH_3)_3$	NH	>100
4e	$-CH(CH_3)CH_2CH_3$	$-C(CH_3)_3$	NH	>100
4f	$-CH_2Ph$	$-C(CH_3)_3$	NH	>100
4g	$-CH_2CH_2SCH_3$	$-C(CH_3)_3$	NH	>100
4h	$-CH_2CH(CH_3)_2$	$-CH_2Ph$	O	9.1 ± 0.78
4i	$-CH_2CH(CH_3)_2$	$-CH_2CH_2Ph$	O	18 ± 2.2
12a	–H	$-CH_2Ph$	NH	32 ± 2.0
12b	$-CH_3$	$-CH_2Ph$	NH	21 ± 1.6
12c	$-CH(CH_3)_2$	$-CH_2Ph$	NH	17 ± 1.2
12d	$-CH(CH_3)CH_2CH_3$	$-CH_2Ph$	NH	4.1 ± 0.51
12e	$-CH_2Ph$	$-CH_2Ph$	NH	5.6 ± 0.60
12f	$-CH_2CH_2SCH_3$	$-CH_2Ph$	NH	3.8 ± 0.28
12g	–H	$-CH_2CH_2Ph$	NH	52 ± 7.2
12h	$-CH_3$	$-CH_2CH_2Ph$	NH	21 ± 2.8
12i	$-CH(CH_3)_2$	$-CH_2CH_2Ph$	NH	>100
12j	$-CH_2CH(CH_3)_2$	$-CH_2CH_2Ph$	NH	1.1 ± 0.12
12k	$-CH(CH_3)CH_2CH_3$	$-CH_2CH_2Ph$	NH	>100
12l	$-CH_2Ph$	$-CH_2CH_2Ph$	NH	6.5 ± 0.48
12m	$-CH_2CH_2SCH_3$	$-CH_2CH_2Ph$	NH	>100
12n	$-CH_2CH(CH_3)_2$	Cyclohexyl	NH	>100
14a	$-CH_2CH(CH_3)_2$		NH	>100
14b	$-CH_2Ph$		NH	>100
Bestatin	—	—	—	8.1 ± 0.60

[a] All of the compounds were assayed three times, and their inhibition results are expressed as the mean values with standard deviations.

among **12a**–**12m**, compounds with benzyl seemed to be better than the ones with phenethyl. Compounds with *t*-butyl (**4a**–**4g**) and cyclohexyl group (**12n**) as R_2 displayed quite low inhibitory activity. For the compounds with two amino acid hydroxamates (**14a** and **14b**) linked together with ureido group only had significantly decreased inhibitory activity. Besides, the amino acid residues also influenced the inhibitory potency. Among **12a**–**12m**, the ones with L-Leucine, L-Isoleucine, L-Phenylalanine and L-Methionine residues showed better activities than the ones with L-Glycine, L-Alanine and L-Valine residues. It demonstrated that the amino acid residues with a comparatively larger side chains might be more beneficial, while **12k** and **12m** were much less potent. Among them L-Leucine residue seemed to be the best one, because **12j** showed to be better than others. As for the linkers, carbamates (**4h** and **4i**) were not as effective as their ureido counterparts, but still showed similar activity to Bestatin in low micromolar range. Compound **12j** was the most active one in all of those compounds (IC_{50} = 1.1 μM), near to eightfold better than Bestatin.

In this preliminary screening, compound **12d, 12e**, **12f**, **12j**, and **12l** showed better activity than Bestatin as the positive control. Hence, their APN inhibitory activities were tested towards MMP-2 (Recombinant, Sigma) and human APN (hAPN) on the surface of intact ES-2 human ovarian clear cell carcinoma cells.

3.1.2. Enzyme inhibitory activity of 12d, 12e, 12f, 12j and 12l towards MMP-2 (Recombinant, Sigma)

Matrix metalloproteinase-2 (MMP-2) is also a zinc dependent metalloproteinase and proved to be closely related with cancer. In order to determine the enzyme selectivity, the active compounds **12d, 12e, 12f, 12j and 12l**, were test for their inhibitory activities towards MMP-2. The result showed that the classical APN inhibitor, Bestatin, exhibited a weak inhibitory potency towards MMP-2 with IC_{50} value of 156 μM. But all the active compounds could not possess obvious inhibition even at the concentration of 500 μM (Table 2).

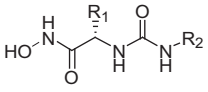
3.1.3. Enzyme inhibitory activity of compound 12d, 12e, 12f, 12j and 12l towards APN on the surface of ES-2 human ovarian cancer cells

Before the examination of the inhibitory effect of APN inhibitors on ES-2 cell proliferation and invasion, their APN inhibitory potency using ES-2 cell was evaluated first. Most of the compounds showed similar inhibitory activity to both of porcine APN and human APN, except **12l**. Compounds **12d**, **12e**, **12f**, and **12j** still displayed better activity than Bestatin, among which **12j** was the most potent one with its IC_{50} value of 5.1 μM, compared with Bestatin of 40 μM (Table 3).

3.2. In vitro cell proliferation assay of 12d, 12e, 12f, and 12j

Furthermore, the anti-proliferation effects of the potent compounds **12d, 12e, 12f,** and **12j** on cancer cells were tested against ES-2 cells (MTT method). As shown in Table 4, the most potent compound **12j** showed much better anti-proliferative effect than

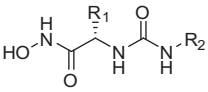
Table 2
The structures and IC_{50} values of the target compoundsa towards MMP-2 (Recombinant, Sigma)



Compounds	R_1	R_2	IC_{50} (μM) towards MMP-2[a]
12d	$-CH(CH_3)CH_2CH_3$	$-CH_2Ph$	>500
12e	$-CH_2Ph$	$-CH_2Ph$	>500
12f	$-CH_2CH(CH_3)_2$	$-CH_2CH_2Ph$	>500
12j	$-CH_2Ph$	$-CH_2CH_2Ph$	>500
12l	$-CH_2Ph$	$-CH_2CH_2Ph$	>500
Bestatin	—	—	156

[a] The compounds were assayed three times, and the standard deviations are <10% of the means.

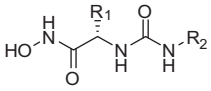
Table 3
The structures and IC_{50} values of some target compounds towards APN on the surface of ES-2



Compounds	R_1	R_2	IC_{50} (μM) towards APN on the surface of ES-2[a]
12d	$-CH(CH_3)CH_2CH_3$	$-CH_2Ph$	39 ± 4.0
12e	$-CH_2Ph$	$-CH_2Ph$	16 ± 1.3
12f	$-CH_2CH_2SCH_3$	$-CH_2Ph$	22 ± 3.0
12j	$-CH_2CH(CH_3)_2$	$-CH_2CH_2Ph$	5.1 ± 0.41
12l	$-CH_2Ph$	$-CH_2CH_2Ph$	110 ± 8.1
Bestatin	—	—	40 ± 3.8

[a] All of the compounds were assayed three times, and their inhibition results are expressed as the mean values with standard deviations.

Table 4
The structures and cell proliferation IC_{50} values of some target compounds towards ES-2



Compounds	R_1	R_2	IC_{50} (μM) towards ES-2[a]
12d	$-CH(CH_3)CH_2CH_3$	$-CH_2Ph$	770
12e	$-CH_2Ph$	$-CH_2Ph$	510
12f	$-CH_2CH_2SCH_3$	$-CH_2Ph$	>1000
12j	$-CH_2CH(CH_3)_2$	$-CH_2CH_2Ph$	96
Bestatin	—	—	490

[a] The compounds were assayed three times, and the standard deviations are <10% of the means.

Bestatin. **12d** and **12e** showed moderate activity, similar to Bestatin. There was only slight anti-proliferation observed in **12f** at the assay concentration.

3.3. Anti-invasion assay

3.3.1. Cell migration assay of 12d, 12e, and 12j

Cancer cell migration as one step of the cancer cell invasion process, was firstly tested using transwell chambers without Matrigel coating. From Figure 3 we found that compounds **12d**, **12e**, **12j** as well as the positive control Bestatin, had no significant inhibitory effects on ES-2 cell migration, although **12d** even had some increase tendency.

We then examined whether the selected APN inhibitors would affect ES-2 cell migration using transwell chambers without Matrigel coating. In this assay, **12d**, **12e**, and **12j** were selected, and we found that those compounds as well as the positive control Bestatin, had no significant inhibitory effect on ES-2 cell migration, although **12d** even had some increase tendency, as shown in Figure 3.

3.3.2. Anti-invasion assay of 12d and 12k

Based on the results of enzyme activity assay towards APN on the ES-2 cell surface, we selected the most potent compounds **12j** to be assayed for anti-invasion effect on ES-2 cell. This assay was performed on transwell chambers coating with Matrigel. The results shown in Figure 4 demonstrated that ES-2 cells freely invaded Matrigel and passed through the chamber. Bestatin, and **12j** could moderately inhibit ES-2 invasion at lower concentration and significantly blocked ES-2 cell invasion at higher concentration. Compound **12j** performed more significant inhibitory effect than Bestatin (P <0.01), yielding 55% inhibition rate versus the control.

From those assays, compound **12j** was the most potent compound with much better inhibitory activities towards both types of APN from porcine kidney and ES-2 cell surface. Compound **12j** exhibited more potent anti-proliferation effect against ES-2 cell. Besides, without obvious influence on ES-2 cell migration, **12j** was able to significantly block ES-2 cell invasion in a dose-dependent manner.

3.4. Statistical analysis

The significance of differences between means was assessed by Student's t test. P Values of <0.05 were considered as statistically significant.

4. Conclusion

A series of novel potent amino acid ureido derivative APN inhibitors with low micromolar enzyme inhibitory activity were designed and synthesized, twelve of which were identified to have the IC_{50} values in a low micromolar range. Among them, compound **12j** showed the most potent APN inhibitory effects (IC_{50} = 1.1 μM). It also displayed significant anti-proliferation and anti-invasion effects in vitro, even more potent than Bestatin. These results suggest that the amino acid ureido derivatives as APN inhibitors could be potential lead compounds to be modified and developed as anticancer proliferation and metastasis agents.

5. Experimental part

5.1. Chemistry

The chemical materials were purchased from commercial suppliers and used without further purification. Solvents were dried

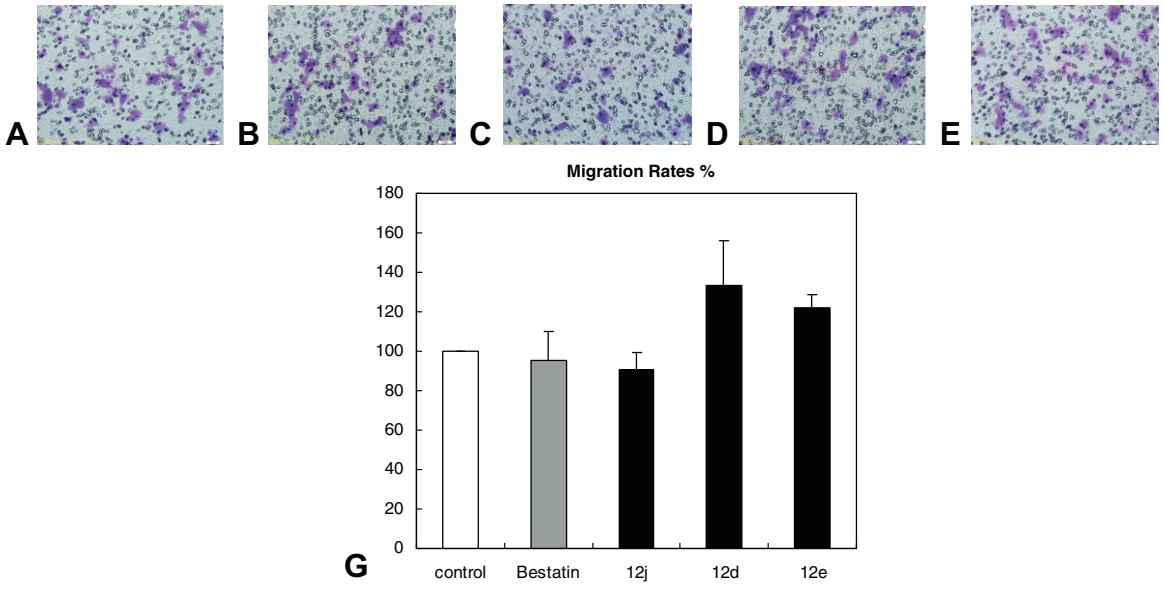


Figure 3. The effects of **12d**, **12e**, and **12j** on the migration of ES-2 cells in vitro. (A) Control, (B) Bestatin 100 μg/mL, (C) **12d** 100 μg/mL, (D) **12e** 100 μg/mL, (E) **12j** 100 μg/mL, and (F) the migration rates of the target compounds on ES-2. Each column represents the mean values with SD for three independent experiments.

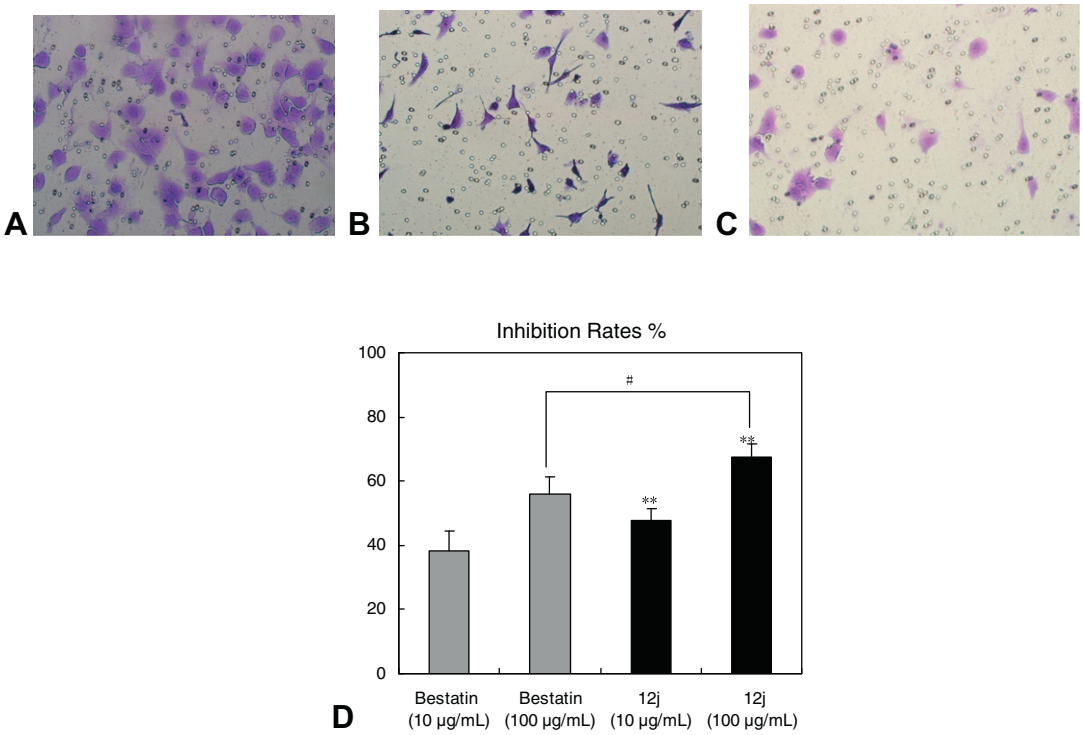


Figure 4. The inhibitory effects of **12d** and **12k** on the invasion of ES-2 cells in vitro. (A) Control, (B) Bestatin 100 μg/mL, (C) **12j** 100 μg/mL, and (D) the invasion inhibition rates of the target compounds on ES-2. Each column represents the mean values with SD values for three independent experiments. $^{**}P$ <0.01, versus the control. $^{\#}P$ <0.05, versus Bestatin treated groups.

over $MgSO_4$ or distilled prior to use and flash chromatography was performed using silica gel (60 Å, 200 ± 300 mesh). The yields are final purified yields. Melting points are uncorrected. NMR spectra were recorded on a Bruker DRX-300 or a Bruker DRX-600 spectrometer. Chemical shifts are in parts per million (ppm). ESI-MS were determined on an API 4000 spectrometer.

5.2. General procedure for the synthesis of 4a–4g

To a mixture of L-Glycine methyl ester hydrochloride (2.63 g, 21 mmol) or the other six amino acid (L-Ala, L-Val, L-Leu, L-Ile, L-Phe, and L-Met) methyl ester hydrochlorides (21 mmol), respectively, in saturated $NaHCO_3$ (80 mL) and DCM (80 mL) was added triphosgene (2.08 g, 7 mmol). The reaction mixture was vigorously stirred under ice-water bath for 15 min and the organic layer was collected. The water layer was extracted with DCM for three times and the organic phase was combined and dried with $MgSO_4$. After the solvent removed under vacuum, the residue was dissolved in DCM (20 mL). This solution was then added to the mixture of *t*-butylamine (1.46 g, 21 mmol) and triethylamine (2.12 g, 21 mmol) in DCM (80 mL) under ice bath. The reaction mixture was stirred at room temperature for 30 min and then the solvent was removed under low pressure. The residue was taken up with ethyl acetate (40 mL) and washed with 1 N HCl (10 mL) and brine (20 mL). The organic phase was dried with $MgSO_4$. After the solvent removed, the residue without purification was directly added to a solution of potassium hydroxylamine (8.37 g, 56 mmol) in methanol (20 mL). The reaction mixture was stirred at room temperature for 5 h and then removed methanol under low pressure. The residue was taken up with 1 N HCl and extracted with ethyl acetate. The organic phase was washed with brine and dried with $MgSO_4$. After the solvent removed under low pressure, the residue was separated by silica gel column chromatography to afford **4a–4g**, respectively.

5.2.1. 2-(3-*tert*-Butyl-ureido)-*N*-hydroxy-acetmaide (4a)

White powder. Yield 65%, R_f = 0.28 (petrol ether/EtOAc, 1:1); mp = 175–177 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.47 (s, 1H), 8.77 (s, 1H), 5.95 (s, 1H), 5.85 (s, 1H), 3.48 (s, 2H), 1.20 (s, 9H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 167.0, 157.1, 49.0, 40.2, 29.2; HRMS-ESI (+) calcd for $C_7H_{15}N_3O_3Na$, 212.1011; found, 212.0991 $[M+Na]^+$.

5.2.2. (*S*)-2-(3-*tert*-Butyl-ureido)-*N*-hydroxy-propionamide (4b)

White powder. Yield 60%, R_f = 0.32 (petrol ether/EtOAc, 1:1); mp = 107–109 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.57 (s, 1H), 8.79 (s, 1H), 5.87 (s, 2H), 4.03 (q, 1H, J = 7.2 Hz), 1.19 (s, 9H), 1.10 (d, 3H, J = 7.2 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 170.1, 156.4, 48.9, 45.7, 29.2, 19.8; HRMS-ESI (+) calcd for $C_8H_{15}N_2O_2$, 171.1133; found, 171.1131 $[M-NHOH]^+$.

5.2.3. (*S*)-2-(3-*tert*-Butyl-ureido)-*N*-hydroxy-2-isopropyl-acetmaide (4c)

White powder. Yield 55%, R_f = 0.38 (petrol ether/EtOAc, 1:1); mp = 138–140 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.58 (s, 1H), 8.79 (s, 1H), 5.91 (s, 1H), 5.78 (d, 1H, J = 9 Hz), 3.80–3.77 (m, 1H), 1.75–1.72 (m, 1H), 1.19 (s, 9H), 0.81 (6H, d, J = 6.6 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.7, 156.8, 55.2, 48.9, 31.4, 29.2 19.1, 18.2; HRMS-ESI (+) calcd for $C_{10}H_{22}N_3O_3$, 232.1661; found, 232.1653 $[M+H]^+$.

5.2.4. (*S*)-2-(3-*tert*-Butyl-ureido)-*N*-hydroxy-2-(2-methyl)-propyl-acetmaide (4d)

White powder. Yield 49%, R_f = 0.46 (petrol ether/EtOAc, 1:1); mp = 124–126 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.62 (s, 1H), 8.86 (s, 1H), 5.88 (s, 1H), 5.85–5.83 (m, 1H), 4.01 (dd, 1H, J = 6.6 Hz, 12.6 Hz), 1.56–1.45 (m, 1H), 1.30–1.27 (m, 2H), 1.19 (s, 9H), 0.85 (d, 6H, J = 6.9 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ

169.5, 156.6, 48.9, 48.6, 42.6, 29.2, 24.2, 22.7, 22.3; HRMS-ESI (+) calcd for $C_{11}H_{25}N_3O_3$, 246.1818; found, 246.1830 $[M+H]^+$.

5.2.5. (*S*)-2-(3-*tert*-Butyl-ureido)-*N*-hydroxy-2-(1-methyl)-propyl-acetmaide (4e)

White powder. Yield 47%, R_f = 0.46 (petrol ether/EtOAc, 1:1); mp = 110–113 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.58 (s, 1H), δ 8.78 (s, 1H), δ 5.89–5.86 (m, 2H), δ 3.85–3.79 (m, 1H), δ 1.55–1.36 (m, 2H), δ 1.19 (s, 1H), δ 0.84–0.77 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.7, 156.7, 54.2, 48.9, 37.7, 29.2, 24.5, 15.3, 11.2; HRMS-ESI (+) calcd for $C_{11}H_{25}N_3O_3$, 246.1818; found, 246.1821 $[M+H]^+$.

5.2.6. (*S*)-2-(3-*tert*-Butyl-ureido)-*N*-hydroxy-2-benzyl-acetmaide (4f)

White powder. Yield 52%, R_f = 0.50 (petrol ether/EtOAc, 1:1); mp = 107–109 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.61 (s, 1H), 8.82 (s, 1H), 7.27–7.16 (m, 5H), 5.95–5.94 (m, 2H), 5.86 (s, 1H), 4.21 (d, 1H, J = 7.2 Hz), 2.83 (dd, 1H, J = 7.2 Hz, 13.5 Hz), 2.68 (d, 1H, J = 7.2 Hz, 13.5 Hz), 1.16 (s, 9H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.6, 156.5, 137.7, 129.2, 128.0, 126.1, 51.5, 48.9, 29.2; HRMS-ESI (+) calcd for $C_{14}H_{21}N_3O_3Na$, 302.1481; found, 302.1461 $[M+Na]^+$.

5.2.7. (*S*)-2-(3-*tert*-Butyl-ureido)-*N*-hydroxy-2-(2-methylsulfanyl)ethyl-acetmaide (4g)

White powder. Yield 51%, R_f = 0.45 (petrol ether/EtOAc, 1:1); mp = 125–127 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ 10.68 (s, 1H), 8.88 (s, 1H), 6.00 (s, 1H), 5.91 (s, 1H), 4.09 (dd, 1H, J = 4.2 Hz, 14.4 Hz), 2.56–2.38 (m, 2H), 2.08 (s, 3H), 1.81–1.77 (m, 1H), 1.71–1.67 (m, 1H), 1.21 (s, 9H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.8, 156.6, 49.5, 49.0, 33.5, 29.4, 29.2, 14.7; HRMS-ESI (+) calcd for $C_{10}H_{21}N_3O_3SNa$, 286.1201; found, 286.1203 $[M+Na]^+$.

5.3. General procedure for the synthesis of 4h, 4i, 14a and 14b

To a mixture of L-Leucine methyl ester hydrochloride (3.81 g, 21 mmol, for **4h, 4i,** and **14a**) or L-Phenylalanine methyl ester hydrochlorides (4.52 g, 21 mmol, for **14b**), respectively, in saturated $NaHCO_3$ (80 mL) and DCM (80 mL) was added triphosgene (2.08 g, 7 mmol). The reaction mixture was vigorously stirred under ice-water bath for 15 min and the organic layer was collected. The water layer was extracted with DCM for three times and the organic phase was combined and dried with $MgSO_4$. After the solvent removed under vacuum, the residue was dissolved in DCM (20 mL). This solution was then added to the mixture of benzyl alcohol (2.16 g, 21 mmol, for **4h**), phenylethanol (2.46 g, 21 mmol, for **4i**), L-Leucine methyl ester hydrochloride (3.81 g, 21 mmol, for **14a),** or L-Phenylalanine methyl ester hydrochlorides (4.52 g, 21 mmol, for **14b**), respectively, and triethylamine (2.12 g, 21 mmol) in DCM (80 mL) under ice bath. The reaction mixture was stirred at room temperature for 30 min and then the solvent was removed under low pressure. The residue was taken up with ethyl acetate (40 mL) and washed with 1 N HCl (10 mL) and brine (20 mL). The organic phase was dried with $MgSO_4$. After the solvent removed, the residue without purification was directly added to a solution of potassium hydroxylamine (8.37 g, 56 mmol) in methanol (20 mL). The reaction mixture was stirred at room temperature for 5 h and then removed methanol under low pressure. The residue was taken up with 1 N HCl and extracted with ethyl acetate. The organic phase was washed with brine and dried with $MgSO_4$. After the solvent removed under low pressure, the residue was separated by silica gel column chromatography to afford **4h**, **4i**, **14a** and **14b**, respectively.

5.3.1. (*S*)-(1-Hydroxycarbamoyl-3-methyl-butyl)-carbamic acid benzyl ester (4h)

White powder. Yield 39%, R_f = 0.35 (petrol ether/EtOAc, 2:1); mp = 86–88 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.65 (s, 1H), 8.79 (s, 1H), 8.30 (s, 1H), 7.42–30 (m, 5H), 5.01 (s, 2H), 4.08–4.03 (m, 1H), 1.58–1.38 (m, 3H), 0.90–0.82 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.8, 155.7, 137.0, 128.3, 127.7, 127.6, 65.3, 50.8, 40.8, 24.1, 22.7, 21.6; HRMS-ESI (+) calcd for $C_{14}H_{21}N_2O_4$, 281.1501; found, 281.1487 $[M+H]^+$.

5.3.2. (*S*)-(1-Hydroxycarbamoyl-3-methyl-butyl)-carbamic acid phenethyl ester (4i)

White powder. Yield 41%, R_f = 0.40 (petrol ether/EtOAc, 2:1); mp = 88–90 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 8.78 (s, 1H), 7.32–7.18 (m, 6H), 4.15–4.11 (m, 2H), 3.95–3.85 (m, 1H), 2.87–2.83 (m, 2H), 1.59–1.24 (m, 3H), 0.87–0.82 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.9, 155.8, 138.2, 128.8, 128.3, 126.2, 64.4, 50.7, 40.8, 34.8, 24.1, 22.7, 21.6; HRMS-ESI (+) calcd for $C_{15}H_{23}N_2O_4$, 295.1658; found, 295.1656 $[M+H]^+$.

5.3.3. (*S,S*)-2-[3-(1-Hydroxycarbamoyl-3-methyl-butyl)-ureido]-4-methyl-pentanoic acid hydroxyamide (14a)

White powder. Yield 24%, R_f = 0.32 (petrol ether/EtOAc, 1:2); mp = 170–173 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.65 (s, 1H), 8.80 (s, 1H), 6.20 (d, 1H, J = 5.7 Hz), 4.07–3.99 (m, 1H), 1.54–1.32 (m, 3H), 0.88–0.83 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.7, 154.4, 52.6, 23.9, 23.0, 21.4; HRMS-ESI (+) calcd for $C_{13}H_{27}N_4O_5$, 319.1982; found, 319.1993 $[M+H]^+$.

5.3.4. (*S,S*)-*N*-Hydroxy-2-[3-(1-hydroxycarbamoyl-2-phenyl-ethyl)-ureido]-3-phenyl-propionamide (14b)

White powder. Yield 24%, R_f = 0.38 (petrol ether/EtOAc, 1:2); mp = 155–158 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.71 (s, 1H), 10.56 (s, 1H), 8.93 (s, 1H), 8.81 (s, 1H), 7.32–7.07 (m, 10H), 6.38 (s, 1H), 6.35 (s, 1H), 4.66–4.60 (m, 2H), 4.18–4.16 (s, 1H), 4.10–4.05 (m, 1H), 3.37–3.14 (m, 4H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 173.4, 168.3, 164.9, 155.8, 137.6, 136.3, 129.2, 129.0, 128.2, 128.0, 126.5, 126.4, 57.3, 52.2, 37.7, 33.4; HRMS-ESI (+) calcd for $C_{19}H_{23}N_4O_5$, 387.1668; found, 387.1656 $[M+H]^+$.

5.4. General procedure for the synthesis of 12a–12f

Benzylamine (1.61 g, 15 mmol).was added to a solution of triphosgene (2.22 g, 7.5 mmol) in dry toluene (80 mL) in room temperature. The reaction mixture was refluxed for 4 h and then solvents removed under low pressure. The residue was dissolved in DCM (20 mL) and this solution was added to the mixture of L-Glycine methyl ester hydrochloride (2.64 g, 15 mmol) or the other amino acid (L-Ala, L-Val, L-Ile, L-Phe, and L-Met) methyl ester hydrochlorides (15 mmol), respectively, and triethylamine (1.52 g, 15 mmol) in DCM (80 mL) under ice-bath. After stirred at room temperature for 30 min, the reaction mixture was concentrated under vacuum and then ethyl acetate (20 mL) was added to the residue. The organic phase was washed with 1 N HCl (10 mL) and saturated brine (10 mL) and dried with $MgSO_4$. After the solvent removed under low pressure, the residue without purification was directly added to a solution of potassium hydroxylamine (8.37 g, 56 mmol) in methanol (20 mL). The reaction mixture was stirred at room temperature for 5 h and then removed methanol under low pressure. The residue was taken up with 1 N HCl and extracted with ethyl acetate. The organic phase was washed with brine and dried with $MgSO_4$. After the solvent removed under low pressure, the residue was separated by silica gel column chromatography to afford **12a–12f**, respectively.

5.4.1. 2-(3-Benzyl-ureido)-*N*-hydroxy-acetmaide (12a)

White powder. Yield 68%, R_f = 0.26 (petrol ether/EtOAc, 1:1); mp = 137–139 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.49 (s, 1H), 8.78 (s, 1H), 7.33–7.19 (m, 5H), 6.61 (t, 1H, J = 7.5 Hz), 6.18–6.02 (t, 1H, J = 5.7 Hz), 4.20 (d, 2H, J = 7.5 Hz), 3.57 (d, 2H, J = 5.7 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 166.9, 157.9, 140.6, 128.2, 127.0, 126.5, 42.9, 40.6; HRMS-ESI (+) calcd for $C_{10}H_{24}N_3O_3$, 224.1035; found, 224.1034 $[M+H]^+$.

5.4.2. (*S*)-2-(3-Benzyl-ureido)-*N*-hydroxy-propoinamide (12b)

White powder. Yield 67%, R_f = 0.32 (petrol ether/EtOAc, 1:1); mp = 108–111 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.59 (s, 1H), 8.80 (s, 1H), 7.33–7.19 (m, 5H), 6.48 (t, 1H, J = 6.0 Hz),,6.16 (d, 1H, J = 8.1), 4.19 (d, 2H, J = 6.0 Hz), 4.14–4.04 (m, 1H), 1.14 (d, J = 6.9 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 175.2, 157.5, 140.6, 128.2, 126.9, 126.5, 48.1, 42.7, 18.3; HRMS-ESI (+) calcd for $C_{11}H_{27}N_3O_3$, 238.1192; found, 238.1181 $[M+H]^+$.

5.4.3. (*S*)-2-(3-Benzyl-ureido)-*N*-hydroxy-2-isopropyl-acetamide (12c)

White powder. Yield 68%, R_f = 0.36 (petrol ether/EtOAc, 1:1); mp = 180–183 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.61 (s, 1H), 8.80 (s, 1H), 7.33–7.19 (m, 5H), 6.50 (t, 1H, J = 6.0 Hz), 6.16 (d, 1H, J = 9.3 Hz), 4.20 (d, 2H, J = 6.0 Hz), 3.87 (dd, 1H, J = 7.2 Hz, 9.3 Hz), 1.85–1.74 (m, 1H), 0.85 (d, 6H, 6.9 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.5, 157.6, 140.7, 128.2, 126.9, 126.5, 55.8, 42.8, 31.3, 19.1, 18.2; HRMS-ESI (+) calcd for $C_{13}H_{19}N_3O_3Na$, 288.1324; found, 288.1330 $[M+H]^+$.

5.4.4. (*S*)-2-(3-Benzyl-ureido)-*N*-hydroxy-2-(1-methyl)-propyl-acetamide (12d)

White powder. Yield 55%, R_f = 0.42 (petrol ether/EtOAc, 1:1); mp = 148–150 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.63 (s, 1H), 8.80 (s, 1H), 7.33–7.10 (m, 5H), 6.52–6.45 (m, 1H), 6.18–6.10 (m, 1H), 4.20 (d, 1H, J = 6.0 Hz), 4.06–4.00 (m, 1H), 1.34–1.19 (m, 2H), 1.10–1.08 (m, 1H), 0.92–0.79 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.8, 157.5, 140.7, 128.2, 126.9, 126.5, 54.8, 42.8, 29.3, 25.7, 24.4; HRMS-ESI (+) calcd for $C_{14}H_{22}N_3O_3$, 280.1661; found, 280.1662 $[M+H]^+$.

5.4.5. (*S*)-2-(3-Benzyl-ureido)-*N*-hydroxy-2-benzyl-acetmaide (12e)

White powder. Yield 45%, R_f = 0.46 (petrol ether/EtOAc, 1:1); mp = 140–142 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.64 (s, 1H), 8.82 (s, 1H), 7.31–7.14 (m, 10H), 6.43 (t, 1H, J = 6.0 Hz), 6.24 (d, 1H, J = 5.8 Hz), 4.34–4.22 (m, 1H), 4.15 (d, 2H, J = 6.0 Hz), 2.88 (dd, 1H, J = 6.0 Hz, 15.0 Hz), 2.72 (dd, 1H, J = 6.0 Hz, 15.0 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.8, 157.2, 140.6, 137.7, 129.2, 128.1, 128.0, 126.8, 126.5, 126.2, 52.1, 42.7; HRMS-ESI (+) calcd for $C_{17}H_{20}N_3O_3$, 314.1505; found, 314.1492 $[M+H]^+$.

5.4.6. (*S*)-2-(3-Benzyl-ureido)-*N*-hydroxy-2-(2-methylsulfanyl) ethyl-acetmaide (12f)

White powder. Yield 42%, R_f = 0.40 (petrol ether/EtOAc, 1:1); mp = 153–155 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.68 (s, 1H), 8.86 (s, 1H), 7.33–7.19 (m, 5H), 6.47 (t, 1H, J = 6.0 Hz), 6.27 (d, 1H, J = 6.0 Hz), 4.20 (d, 2H, J = 6.0 Hz), 4.16–4.09 (m, 1H), 2.45–2.34 (m, 2H), 2.03 (s, 3H), 1.85–1.63 (m, 2H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.6, 157.4, 140.6, 128.2, 126.9, 126.6, 50.0, 42.8, 33.3, 29.4, 14.8; HRMS-ESI (+) calcd for $C_{13}H_{20}N_3O_3S$, 298.1225; found, 298.1230 $[M+H]^+$.

5.5. General procedure for the synthesis of 12g–12m

Phenethylamine (1.82 g, 15 mmol).was added to a solution of triphosgene (2.22 g, 7.5 mmol) in dry toluene (80 mL) in room temperature. The reaction mixture was refluxed for 4 h and then solvents removed under low pressure. The residue was dissolved in DCM (20 mL) and this solution was added to the mixture of L-Glycine methyl ester hydrochloride (2.64 g, 15 mmol) or the other amino acid (L-Ala, L-Val, L-Leu, L-Ile, L-Phe, and L-Met) methyl ester hydrochlorides (15 mmol), respectively, and triethylamine (1.52 g, 15 mmol) in DCM (80 mL) under ice-bath. After stirred at room temperature for 30 min, the reaction mixture was concentrated under vacuum and then ethyl acetate (20 mL) was added to the residue. The organic phase was washed with 1 N HCl (10 mL) and saturated brine (10 mL) and dried with $MgSO_4$. After the solvent removed under low pressure, the residue without purification was directly added to a solution of potassium hydroxylamine (8.37 g, 56 mmol) in methanol (20 mL). The reaction mixture was stirred at room temperature for 5 h and then removed methanol under low pressure. The residue was taken up with 1 N HCl and extracted with ethyl acetate. The organic phase was washed with brine and dried with $MgSO_4$. After the solvent removed under low pressure, the residue was separated by silica gel column chromatography to afford **12h–12n**, respectively.

5.5.1. 2-(3-Phenethyl-ureido)-*N*-hydroxy-acetmaide (12g)

White powder. Yield 63%, R_f = 0.28 (petrol ether/EtOAc, 1:1); mp = 125–128 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.53 (s, 2H), 7.32–7.17 (m, 5H), 6.22–6.12 (m, 1H), 6.10–5.98 (m, 1H), 3.86 (d, 2H, J = 4.5 Hz), 3.21 (dd, 2H, J = 6.9 Hz, 12.9 Hz), 2.66 (t, 2H, J = 6.9 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 166.7, 157.9, 139.7, 128.6, 128.2, 125.9, 41.0, 40.8, 36.1; HRMS-ESI (+) calcd for $C_{11}H_{16}N_3O_3$, 238.1192; found, 238.1189 $[M+H]^+$.

5.5.2. (*S*)-2-(3-Phenethyl-ureido)-*N*-hydroxy-propoinamide (12h)

White powder. Yield 64%), R_f = 0.32 (petrol ether/EtOAc, 1:1); mp = 137–139 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.59 (s, 1H), 8.82 (s, 1H), 7.32–7.18 (m, 5H), 6.13 (d, 1H, J = 8.1 Hz), 6.03 (t, 1H, J = 5.4 Hz), 4.10–4.00 (m, 1H), 3.24–3.17 (m, 2H), 2.65 (t, 2H, J = 7.2 Hz), 1.10 (d, 3H, J = 6.9 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.9, 157.1, 139.6, 128.6, 128.2, 125.9, 46.2, 40.8, 36.1, 19.7; HRMS-ESI (+) calcd for $C_{12}H_{17}N_3O_3Na$, 274.1168; found, 274.1154 $[M+Na]^+$.

5.5.3. (*S*)-2-(3-Phenethyl-ureido)-*N*-hydroxy-2-isopropyl-acetamide (12i)

White powder. Yield 47%, R_f = 0.32 (petrol ether/EtOAc, 1:1); mp = 87–90 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.19 (s, 1H), 7.31–7.17 (m, 1H), 3.90–3.88 (m, 1H), 3.67–3.49 (m, 2H), 2.87–2.82 (m, 2H), 1.98–1.90 (m, 1H), 0.93–0.65 (m, 7H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 173.4, 157.0, 138.1, 128.6, 128.3, 126.3, 61.2, 38.6, 33.2, 29.5, 18.4, 15.7; HRMS-ESI (+) calcd for $C_{14}H_{19}N_2O_2$, 247.1446; found, 247.1448 $[M-NHOH]^+$.

5.5.4. (*S*)-2-(3-Phenethyl-ureido)-*N*-hydroxy-2-(2-methyl)-propyl-acetamide (12j)

White powder. Yield 41%, R_f = 0.38 (petrol ether/EtOAc, 1:1); mp = 155–157 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.65 (s, 1H), 8.78 (s, 1H), 6.06 (d, 1H, J = 9.0 Hz), 5.94 (t, 1H, J = 5.7 Hz), 4.05 (dd, 1H, J = 7.5 Hz, 16.2 Hz), 3.21 (dd, 2H, J = 6.9 Hz, 13.2 Hz), 2.65 (t, 2H, J = 7.2 Hz), 1.56–1.34 (m, 1H), 1.31 (t, 2H, J = 7.5 Hz), 0.86 (t, 6H, J = 6.9 Hz); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.5, 157.3, 139.7, 139.6, 128.6, 128.2, 125.9, 49.0, 42.4, 40.8, 24.2, 22.7, 22.3, 22.1; HRMS-ESI (+) calcd for $C_{15}H_{24}N_3O_3$, 294.1818; found, 294.1813 $[M+H]^+$.

5.5.5. (*S*)-2-(3-Phenethyl-ureido)-*N*-hydroxy-2-(1-methyl)-propyl-acetamide (12k)

White powder. Yield 40%, R_f = 0.38 (petrol ether/EtOAc, 1:1); mp = 108–110 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.48 (s, 1H), 8.15 (s, 1H), 7.30–7.18 (m, 5H), 4.00–3.92 (m, 1H), 3.66–3.52 (m, 2H), 2.83 (t, 2H, J = 6.6 Hz), 1.73–1.67 (m, 1H), 1.39–1.29 (m, 1H), 1.20–1.08 (m, 1H), 0.87–0.76 (m, 3H), 0.59–0.57 (m, 3H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 173.8, 157.1, 138.0, 128.6, 128.3, 126.3, 59.7, 35.9, 33.1, 25.4, 12.8, 11.6; HRMS-ESI (+) calcd for $C_{15}H_{21}N_2O_2$,261.1603; found, 261.1606 [M−NHOH]$^+$.

5.5.6. (*S*)-2-(3-Phenethyl-ureido)-*N*-hydroxy-2-benzyl-acetmaide (12l)

White powder. Yield 45%, R_f = 0.45 (petrol ether/EtOAc, 1:1); mp = 133–135 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.62 (s, 1H), 8.81 (s, 1H), 7.30–7.16 (m, 10H), 6.20 (d, 1H, J = 8.7 Hz), 5.99 (t, 1H, J = 5.7 Hz), 4.29–4.21 (m, 1H), 3.20–3.13 (m, 2H), 2.84 (dd, 1H, J = 6.0 Hz, 13.5 Hz), 2.72 (dd, 1H, J = 6.0 Hz, 13.5 Hz), 2.68–2.60 (m, 2H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.6, 157.1, 139.6, 137.7, 129.2, 128.6, 128.2, 128.0, 126.1, 125.9, 52.0, 40.8, 36.0; HRMS-ESI (+) calcd for $C_{18}H_{22}N_3O_3$, 328.1661; found, 328.1664 [M+H]$^+$.

5.5.7. (*S*)-2-(3-Phenethyl-ureido)-*N*-hydroxy-2-(2-methylsulfanyl)ethyl-acetmaide (12m)

White powder. Yield 40%, R_f = 0.40 (petrol ether/EtOAc, 1:1); mp = 138–140 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.65 (s, 1H), 8.78 (s, 1H), 7.32–7.17 (m, 5H), 6.21 (d, 1H, J = 8.7 Hz), 6.00 (s, 1H) 4.12–4.06 (m, 1H), 3.22 (dd, 2H, J = 6.9 Hz, 13.0 Hz), 2.66 (t, 2H, J = 7.2 Hz), 2.42–2.31 (m, 2H), 2.03 (s, 3H), 1.82–1.60 (m, 2H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 168.7, 157.3, 139.6, 128.6, 128.2, 126.9, 50.0, 40.8, 36.0, 33.2, 29.4, 14.6; HRMS-ESI (+) calcd for $C_{14}H_{22}N_3O_3S$, 312.1382; found, 312.1367 [M+H]$^+$.

5.6. General procedure for the synthesis of 12n

Cyclohexylamine (1.47 g, 15 mmol).was added to a solution of triphosgene (2.22 g, 7.5 mmol) in dry toluene (80 mL) in room temperature. The reaction mixture was refluxed for 4 h and then solvents removed under low pressure. The residue was dissolved in DCM (20 mL) and this solution was added to the mixture of L-Leucine methyl ester hydrochloride (2.72 g, 15 mmol) and triethylamine (1.52 g, 15 mmol) in DCM (80 mL) under ice-bath. After stirred at room temperature for 30 min, the reaction mixture was concentrated under vacuum and then ethyl acetate (20 mL) was added to the residue. The organic phase was washed with 1 N HCl (10 mL) and saturated brine (10 mL) and dried with $MgSO_4$. After the solvent removed under low pressure, the residue without purification was directly added to a solution of potassium hydroxylamine (8.37 g, 56 mmol) in methanol (20 mL). The reaction mixture was stirred at room temperature for 5 h and then removed methanol under low pressure. The residue was taken up with 1 N HCl and extracted with ethyl acetate. The organic phase was washed with brine and dried with $MgSO_4$. After the solvent removed under low pressure, the residue was separated by silica gel column chromatography to afford **12n**, white powder, yield 60%, R_f = 0.42 (Petrol Ether/EtOAc, 1:1); mp = 138–141 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.66 (s, 1H), 9.20 (s, 1H), 5.90–5.86 (m, 2H), 4.08–4.02 (m, 1H), 3.33–3.28 (m, 1H), 1.72–1.47 (m, 6H), 1.33–1.02 (m, 7H), 0.99–0.83 (m, 6H); ^{13}C NMR (300 MHz, DMSO-d_6): δ 169.5, 156.6, 48.8, 47.5, 42.6, 33.2, 25.2, 24.3, 24.2, 22.7, 22.2; HRMS-ESI (+) calcd for $C_{13}H_{26}N_3O_3$, 272.1974; found, 272.1969 [M+H]$^+$.

5.7. Biological activity methods. Cell culture

ES-2 cells were cultured in RPMI-1640 culture medium supplemented with 10% (v/v) fetal bovine serum (FBS).

5.7.1. Enzyme inhibitory activity of the amino acid ureido derivatives towards APN from porcine kidney (Microsomal, Sigma)

IC_{50} values against APN were determined by using L-Leu-*p*-nitroanilide as substrate and Microsomal APN from Porcine Kidney Microsomes (Sigma) as lymphilized powder 15–25 units/mg protein. In brief, the assay was performed in 96-well plates in 50 mM PBS, pH 7.2 as the assay buffer, at 37 °C. All solutions of the inhibitors were prepared in the assay buffer with 0.5% DMSO in final concentration as the fluxing agent. All inhibitors were pre-incubated with APN for 5 min at room temperature. The assay mixture, which contained 40 μL of the inhibitor solution (concentration dependent on the inhibitors), 100 μL of the enzyme solution (6 μg/mL in final concentration), 5 μL of the substrate solution and the assay buffer, was adjusted to 200 μL, then incubated at 37 °C for 30 min. 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. The enzyme activity inhibition rate was calculated as $[(OD_c - OD_t)/(OD_c - OD_z)] \times 100$. OD_c represents the OD values of the control group, OD_t represents the OD values of the treating groups, and OD_z represents the OD values of the zero-setting groups. The concentration of inhibiting 50% of the enzyme activity (IC_{50}) was calculated.

5.7.2. Enzyme inhibitory activity of 12d, 12e, 12f, 12j and 12l towards APN on the surface of ES-2 cells

IC_{50} values against APN were determined by using L-Leu-*p*-nitroanilide as substrate and ES-2 human ovarian carcinoma cells as the enzyme source. In brief, the assay was performed in 96-well plates in 10 mM PBS, pH 7.4 as the assay buffer, at 37 °C. All solutions of inhibitors were prepared in the assay buffer with 0.1% DMSO in final concentration as the fluxing agent. Bestatin, **12d, 12e, 12f, 12j** and **12l** were pre-incubated with ES-2 cell suspension (2×10^5 cells per well) for 5 min at room temperature. The assay mixture, which contained 20 μL of the inhibitor solution (concentration dependent on the inhibitors), 70 μL of the ES-2 cell suspension, 10 μL of the substrate solution, or the assay buffer, was adjusted to 100 μL, and then incubated at 37 °C for 1 h. The hydrolysis of the substrate in the supernatant liquor after centrifugation was monitored by following the change in the absorbance measured at 405 nm with the UV–Vis spectrophotometer Pharmacia LKB, Biochrom 4060. The enzyme activity inhibition rate was calculated as $[(OD_c - OD_t)/(OD_c - OD_z)] \times 100$. OD_c represents the OD values of the control group, OD_t represents the OD values of the treating groups, and OD_z represents the OD values of the zero-setting groups. The concentration of inhibiting 50% of the enzyme activity (IC_{50}) was calculated.

5.7.3. Enzyme inhibitory activity of the compounds towards MMP-2 (Recombinant, Sigma)

IC_{50} values against MMP-2 in 96-well microtiter plates using succinylated gelatin as the substrate. The compounds and the enzyme were dissolved in sodium borate buffer (pH 8.5, 50 mM) and incubated at 37 °C for 10 min. The substrate was added and incubated at 37 °C for additional 60 min. The control and zero-setting groups were also carried out. Then 0.03% picrylsulfonic acid solution was added and incubated at room temperature for additional 20 min. The resulting solutions were measured under

450 nm with the UV–Vis spectrophotometer Pharmacia LKB, Biochrom 4060. The inhibitory rates were calculated by $[(OD_c - OD_t)/(OD_c - OD_z)] \times 100$. OD_c represents the OD values of the control group, OD_t represents the OD values of the treating groups, and OD_z represents the OD values of the zero-setting groups. The concentration of inhibiting 50% of the enzyme activity (IC_{50}) was calculated.

5.7.4. Anti-proliferation assay

The anti-proliferation assay of compound **12d**, **12e**, **12f**, and **12j** were assayed by MTT method. In brief, the ES-2 cells were plated in a 96-well plate (8000 cells per well) in RPMI-1640 culture medium with 10% FBS, and allowed to adhere and spread for 10 h. Then the culture medium was removed and of Bestatin, **12d**, **12e**, **12f**, and **12j** at various concentration in RPMI-1640 culture medium with 10% FBS (with 0.1% DMSO as fluxing agent, also in the control the zero-setting wells) were added, and then the cells were cultured for 48 h at 37 °C in a CO_2 incubator. MTT solution (10 μL of 5 mg/mL) was added per well and the cells were cultured for additional 4 h. Then the medium were removed and the residue were dissolved in 100 μL DMSO per well. The optical density (OD) values were measured at 590 nm. The growth inhibition rate was calculated as $[(OD_c - OD_t)/(OD_c - OD_z)] \times 100$. OD_c represents the OD values of the control group, OD_t represents the OD values of the treating groups, and OD_z represents the OD values of the zero-setting groups. The concentration of inhibiting 50% of the enzyme activity (IC_{50}) was calculated.

5.7.5. Cell migration assay

The Thincert™ Chambers (Costar, Cambridge, MA) with 100 μg/mL of **12d, 12e, 12f** and **12j** in 100 μL of RPMI-1640 culture medium with 1% FBS (with 0.1% DMSO in final concentration as the fluxing agents) were added to the upper chamber at the same time. Cells in 400 μL of RPMI-1640 culture medium with 1% FBS (60000 cells per well) were added and allowed to migrate for 3 h at 37 °C in a CO_2 incubator. 3 h later, cells in the upper chamber were removed with a cotton swab. The migrated cells were fixed, stained with 0.1% crystal violet, and photographs were taken under a microscope and measured at 595 nm after extraction with 33% acetic acid. The migration rates were calculated as $[(OD_t - OD_a)/(OD_c - OD_z)] \times 100$. OD_c represents the OD values of the control group, OD_t represents the OD values of the treating groups, and OD_z represents the OD values of the zero-setting groups.

5.7.6. Anti-invasion assay

The BD BioCoat™ Matrigel™ Invasion Chambers (BD Bioscience, Bedford, MA) were rehydrated with 500 μL RPMI-1640 culture medium with 1% FBS in the upper and lower chambers, respectively, for 2 h. After the medium was removed, 750 μL of RPMI-1640 culture medium with 10% FBS were added to the lower chambers. Various concentrations (10 and 100 μg/mL) of **12j** in 100 μL of RPMI-1640 culture medium with 1% FBS (with 0.1% DMSO in final concentration as the fluxing agents) were added to the upper chamber at the same time. Cells in 400 μL of RPMI-1640 culture medium with 1% FBS (100,000 cells per well) were added and allowed to invade for 8 h at 37 °C in a CO_2 incubator. 8 h later, matrigel and cells in the upper chamber were removed with a cotton swab. The remaining cells were fixed, stained with 0.1% crystal violet, and photographs were taken under a microscope. The inhibition rates were quantitated by counting the number of cells in five random fields (×100) per insert.

5.7.7. Statistical analysis

All biological experiments, including the enzyme inhibition assays, the in MTT assay, the cell migration assay, and the anti-invasion assay were performed at least three times with triplicates in each experiment. Representative results are depicted in the report. Data are presented as means ± SD, and comparisons were made using Student' *t* test. A probability of 0.05 or less was considered statistically significant.

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