


Cite this: *RSC Adv.*, 2025, 15, 4348

# Synthesis, biological and pharmacokinetic characterization of a novel leucine ureido derivative as a multi-target anticancer agent†

Fangyuan Shi,<sup>a</sup> Qifu Xu,<sup>b</sup> Yingjie Zhang,<sup>b</sup> Jiangying Cao,<sup>\*c</sup> Chunxi Liu<sup>\*a</sup> and Anchang Liu<sup>\*a</sup>

Previously, a novel series of leucine ureido derivatives containing the 1,2,3-triazole moiety were identified and validated as potent aminopeptidase N inhibitors with marked *in vitro* and *in vivo* antitumor potencies. Moreover, synergistic anti-proliferation effects against tumor cells were found when used in combination with 5-Fluorouracil (5-FU). Herein, a novel leucine ureido derivative (compound **3**) was synthesized by coupling cytotoxic agent 5-FU with leucine ureido derivatives containing the 1,2,3-triazole moiety *via* esterification. The biological activity evaluation showed that compound **3** exhibited more potent *in vitro* anti-proliferative, anti-metastatic, anti-angiogenic activities than the positive control bestatin. Furthermore, it was observed that compound **3** was very stable in simulated gastric fluid, while slowly cleaved in simulated intestinal fluid. *In vivo* pharmacokinetic study displayed that compound **3** was absorbed quickly after oral administration in rats and maintained *in vivo* for a long time, but exhibited poor oral bioavailability. Generally speaking, compound **3** is a promising lead for further development of more potent analogs as anticancer agents.

Received 30th April 2024  
Accepted 17th November 2024

DOI: 10.1039/d4ra03200d

rsc.li/rsc-advances

## 1. Introduction

Aminopeptidase N (APN; CD13; EC 3.4.11.2) belonging to the M1 family of the MA clan of peptidase is a Zn<sup>2+</sup> dependent membrane-bound exopeptidase,<sup>1,2</sup> which consist of a short cytoplasmic domain, a single transmembrane part and a large extra-cellular domain.<sup>3</sup> As a moonlighting protein, APN presents multiple biological functions, such as enzymatic activity, antigen presentation and the receptor for some viruses.<sup>4</sup> It was reported that APN was associated with the tumor migration, invasion, metastasis and angiogenesis.<sup>5–7</sup> Moreover, APN is a functional marker of semi-dormant liver cancer cells which are responsible for chemotherapy resistance and cancer relapse.<sup>8</sup>

Due to the important role of APN in tumors, APN inhibitors (APNIs) had caught the attention of scientists. So far, many APN inhibitors have been reported.<sup>9,10</sup> Bestatin, containing an AHPA skeleton, has displayed diverse biological activities, such as anti-angiogenic, anti-metastatic, and immunomodulatory

effects.<sup>11,12</sup> 5-Fluorouracil (5-FU, compound **1**), a pyrimidine fluoride derivative, presents inhibitory activities against various carcinomas. However, it is limited in clinical application due to the side effects and shortcomings, such as marrow toxicity, short plasma half-life, and poor tumor selectivity.<sup>13</sup> Therefore, the N<sup>1</sup> and N<sup>3</sup> positions of 5-FU were usually modified with biodegradable linkers to generate prodrugs to improve the efficacy and reduce toxicity.<sup>14,15</sup>

In the previous work of our group, we synthesized a number of different series of APN inhibitors, of which the leucine ureido derivatives with the 1,2,3-triazole moiety (compound **2**) exhibited excellent APN inhibitory potency and promising *in vitro* and *in vivo* anti-angiogenic and anti-metastatic effects.<sup>16,17</sup> Notably, when combined with 5-fluorouracil (5-FU), synergistic anti-proliferation effects against several tumor cell lines were exhibited and no significant systemic toxicity was found in a mouse hepatoma H22 tumor transplant model.<sup>16</sup>

According to the multi-target drug design approach,<sup>18,19</sup> we designed a new leucine ureido derivative (compound **3**, Fig. 1) by coupling 5-FU moiety with the carboxyl group of leucine ureido derivatives containing the 1,2,3-triazole group. In detail, the ester linker was liable and could be cleaved by the esterase enzyme. Moreover, the hydroxymethyl-5-FU (compound **4**) could be converted to 5-FU *via* an extremely fast process. Therefore, the hybrid drug was supposed to release cytotoxic agent 5-FU and compound **5** by the enzyme-catalyzed metabolic reactions. In addition, compound **3** might have enhanced antitumor activity due to the aforementioned synergistic anti-

<sup>a</sup>Department of Pharmacy, Qilu Hospital of Shandong University, Jinan, Shandong, 250012, China. E-mail: acleu@126.com; liuchunxi1985@163.com

<sup>b</sup>Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Cheeloo College of Medicine, Shandong University, Jinan, Shandong 250012, P. R. China

<sup>c</sup>School of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan, Shandong 250355, P. R. China. E-mail: 60030103@sducm.edu.cn

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4ra03200d>

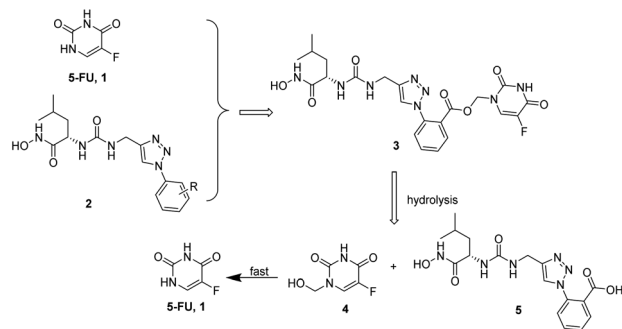



Fig. 1 Design strategy and proposed degradation pathway for the target compound.

proliferation effects of 5-FU combined with the leucine ureido derivatives against tumor cells.<sup>16</sup> Thereby, the *in vitro* anti-proliferative activities, stability and *in vivo* pharmacokinetic properties of compound 3 are reported in this paper.

## 2. Results and discussion

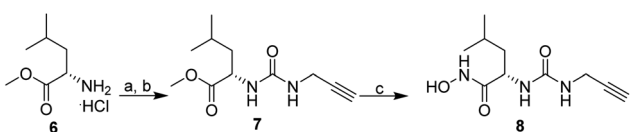
### 2.1. Chemistry

The target compound 3 is synthesized following the routes in Schemes 1 and 2. As shown in Scheme 1, compound 6 reacted with triphosgene to generate isocyanate, which was then immediately reacted with propargylamine to yield the ureido derivative 7. Then,  $\text{NH}_2\text{OK}$  transformed the methyl ester group of 7 to hydroxamate moiety to give the key intermediate 8.

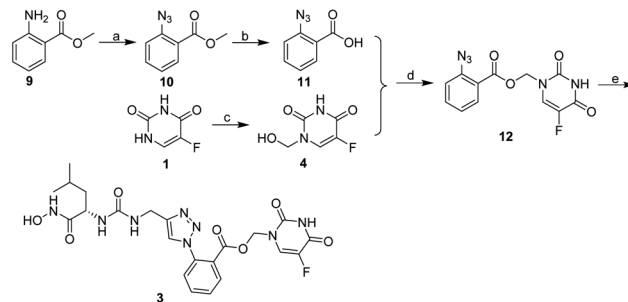
As shown in Scheme 2, activation of the amino groups of the aromatic amine 9 with sodium nitrite in 10% hydrochloric acid and then reacted with sodium azide led to the azide derivative 10, which was hydrolyzed with sodium hydroxide to obtain carboxyl derivative 11. 5-Fluorouracil (1) was reacted with 37% oxy-methylene to give the hydroxymethylated intermediate 4, which was coupled with 11 *via* esterification of carboxyl and hydroxyl group to give intermediate 12. Finally, the target compound 3 was generated by coupling 12 with 8 *via* click chemistry.

### 2.2. Enzyme inhibitory activity of the target compound against porcine APN

The target compound 3 synthesized was firstly evaluated for the inhibitory activity against porcine APN with bestatin as the positive control. The results in Table 1 showed that the APN inhibitory activity of compound 3, with the  $\text{IC}_{50}$  value of  $0.50 \pm 0.2 \mu\text{M}$ , was over 8-fold more potent than the positive control bestatin ( $\text{APN IC}_{50} = 4.08 \pm 0.35 \mu\text{M}$ ).



Scheme 1 Reagents and conditions: (a) triphosgene,  $\text{NaHCO}_3$ ,  $\text{DCM}/\text{H}_2\text{O}$ ,  $0^\circ\text{C}$ , 1.5 h; (b) propargylamine, TEA,  $\text{DCM}$ ,  $25^\circ\text{C}$ , 12 h; (c)  $\text{NH}_2\text{OK}$ ,  $\text{MeOH}$ ,  $25^\circ\text{C}$ , 0.5 h.



Scheme 2 Reagents and conditions: (a)(i) 10%  $\text{HCl}$ ,  $\text{NaNO}_2$ ,  $0^\circ\text{C}$ , 0.5 h, (ii)  $\text{NaN}_3$ ,  $0^\circ\text{C}$ , 0.5 h; (b)  $\text{NaOH}$ ,  $\text{H}_2\text{O}$ ,  $\text{MeOH}$ ,  $25^\circ\text{C}$ , 3 h; (c) 37% oxy-methylene,  $70^\circ\text{C}$ , 2 h; (d) PyBOP,  $\text{Et}_3\text{N}$ ,  $\text{DCM}$ ,  $\text{THF}$ , 24 h; (e) 8,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , sodium ascorbate,  $\text{DMSO}/\text{H}_2\text{O}$  (4 : 1),  $25^\circ\text{C}$ , 5 h.

Table 1 The  $\text{IC}_{50}$  value of the target compound against APN from porcine kidney

Compd	$\text{IC}_{50}^a$ ( $\mu\text{M}$ )
3	$0.50 \pm 0.29$
Bestatin	$4.08 \pm 0.35$

<sup>a</sup> Assays were performed in triplicate; data are shown as mean  $\pm$  SD.

### 2.3. *In vitro* anti-proliferative activities of the target compound against tumor cells

Compound 3 was further evaluated in the anti-proliferation assay against six tumor cell lines (MDA-MB-231, HEL, K562, HuH7, PLC/PRF/5 and HepG2). The results are listed in Table 2. Compared with other tumor cells, HEL and K562 cell lines were more sensitive to compound 3, with the  $\text{IC}_{50}$  values of  $47.18 \pm 19.29 \mu\text{M}$  and  $25.58 \pm 12.31 \mu\text{M}$ , respectively. Moreover, compound 3 presented better anti-proliferative potencies than bestatin against HEL and K562 cell lines.

### 2.4. *In vitro* and *ex vivo* anti-angiogenesis assays

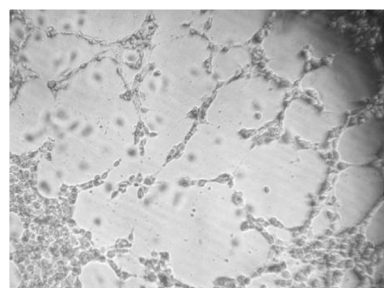
To evaluate the *in vitro* anti-angiogenic potency of compound 3, the human umbilical vein endothelial cells (HUVECs) tubular structure formation assay was performed. The results in Fig. 2 showed that compound 3 could inhibit the capillary tube formation at the concentration of  $5 \mu\text{M}$ . Moreover, compared with  $10 \mu\text{M}$  of bestatin,  $5 \mu\text{M}$  of compound 3 presented less tubular structure formed by HUVECs, indicating the better anti-angiogenesis activity.

It is well-known that the rat aortic ring model could simulate the *in vivo* angiogenesis environment better than the HUVECs tube formation model. The *ex vivo* rat thoracic aorta ring assay was used to further evaluate the anti-angiogenic activity of compound 3. The results are shown in Fig. 3. Compared with the control group, compound 3 could prevent the micro-vessel growth at the concentration of  $5 \mu\text{M}$ . Moreover, similar with the results shown in the HUVECs tubular structure formation assay,  $5 \mu\text{M}$  of compound 3 demonstrated much better inhibitory potency against micro-vessel growth relative to  $10 \mu\text{M}$  of bestatin.

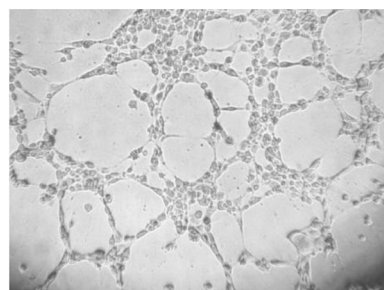
Table 2 The IC<sub>50</sub> values of target compound against proliferation of the tumor cell lines

Compd	IC <sub>50</sub> <sup>a</sup> (μM)					
	MDA-MB-231	HEL	K562	HuH7	PLC/PRF/5	HEPG2
<b>3</b>	>100	47.18 ± 19.29	25.58 ± 12.31	>100	>100	>100
Bestatin	>100	>100	>100	>100	>100	>100

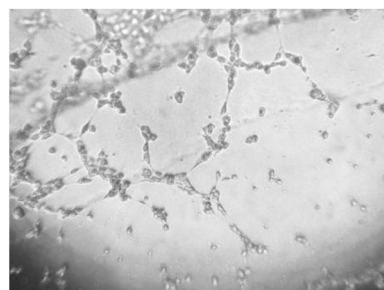
<sup>a</sup> Assays were performed in triplicate; data are shown as mean ± SD.



Ctrl

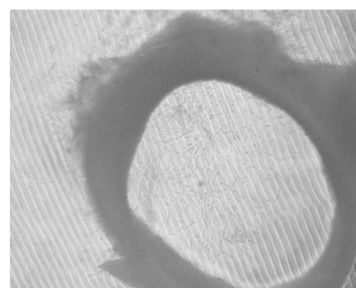


Bestatin (10 μM)

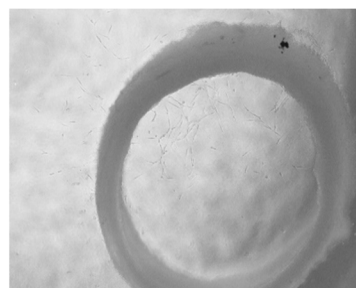


Comp. 3 (5 μM)

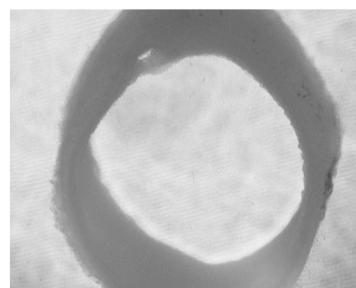
Fig. 2 Representative images of compound **3** on the formation of HUVECs capillary tube-like structure.



Ctrl



Bestatin (10 μM)



Comp. 3 (5 μM)

Fig. 3 Representative images of compound **3** on the micro-vessels growth of the rat aortic ring.

### 2.5. Stability of the test compound *in vitro*

The stability of compound **3** in simulated gastric fluid and simulated intestinal fluid was conducted using a LC/MS/MS method. Briefly, the compound **3** was incubated at 37 °C for the appointed time. At predetermined time points, a sample of the mixture was removed, mixed, centrifuged and analyzed by LC/MS/MS. It was observed that compound **3** was very stable in simulated gastric fluid, while slowly cleaved in simulated intestinal fluid (Fig. 4). The results showed that compound **3** could release significant levels of compound **5** and 5-FU *in vitro*.

Therefore, the anti-proliferative activities of compound **3** against tumor cells might be partly due to the synergistic effect of compound **5** and 5-FU.

### 2.6. Metabolic stability in mouse liver microsomes (MLMs)

Metabolic stability assay of compound **3** with testosterone as the positive control, was performed in liver microsomes of mouse at the concentration of 1 μM. The metabolism of compound **3** in mouse liver microsomes was determined for



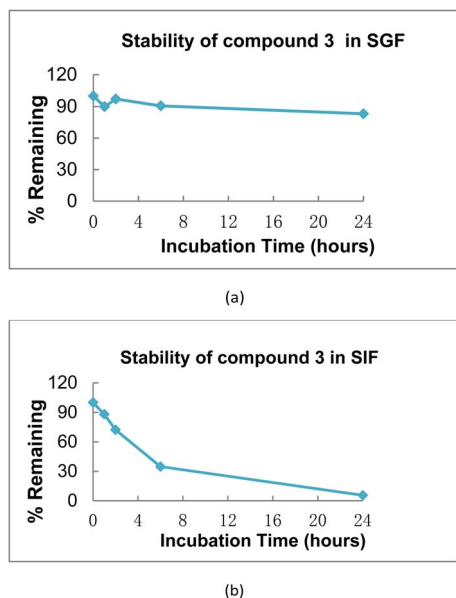


Fig. 4 (a) Stability of compound 3 in simulated gastric fluid, points were achieved after 0, 1, 2, 6, 24 h, respectively; (b) stability of compound 3 in simulated intestinal fluid, points were achieved after 0, 1, 2, 6, 24 h, respectively.

phase I oxidative reactions. The results were listed in Table 3. Compound 3 was stable in MLMs with  $T_{1/2} = 94.9$  min and  $CL_{int}(liver) = 57.8$  mL min<sup>-1</sup> kg<sup>-1</sup>. The remaining amount of compound 3 at 60 min was 69.4% in MLMs, comparing with the group with no co-factor, which indicated that NADPH was involved in the metabolism of the target compound.

### 2.7. *In vivo* PK studies

Given its promising results in the *in vitro* studies, the *in vivo* pharmacokinetic properties of compound 3 were assayed after a single intravenous (i.v.) or oral (p.o.) administration, respectively, which could be beneficial for the further preclinical studies.

The degradation kinetics of compound 3 *in vivo* was evaluated in Sprague Dawley rats (Table 4 and Fig. 5), and LC-MS/MS analysis was conducted to quantitatively determine the plasma concentration of compound 3 and the corresponding metabolites. As shown in Table 4, it was revealed that the mean values of the maximum plasma concentration ( $C_{max}$ ) of compound 3 after i.v. and p.o. administration were  $47\,400 \pm 15\,838$  ng mL<sup>-1</sup> and  $337 \pm 158$  ng mL<sup>-1</sup>, respectively. The areas under the plasma concentration–time curve extrapolated to the last time

point ( $AUC_{0-t}$ ) after i.v. and p.o. administration were  $16\,059 \pm 6837$  ng h mL<sup>-1</sup> and  $484 \pm 119$  ng h mL<sup>-1</sup>, respectively. Moreover, the plasma  $T_{1/2}$  values of compound 3 were  $0.867 \pm 0.387$  h (i.v.) and  $4.44 \pm 4.1$  h (p.o.), respectively. The time to  $C_{max}$  ( $T_{max}$ ) was 0.417 h, indicating that compound 3 was absorbed rapidly after oral administration.

As shown in Fig. 5, after administration of compound 3, its degradation products 5-FU and compound 5 were founded almost simultaneously. Therefore, it was speculated that compound 3 was rapidly metabolized to release 5-FU and compound 5. Although absorbed quickly in rats and maintained *in vivo* for a long time, the absolute oral bioavailability of compound 3 was determined to be 0.64%. Therefore, the attention could be focused on the optimal delivery system to increase the oral bioavailability of compound 3 and enhance the pharmacological efficacy.

## 3. Experimental

### 3.1. Chemistry: general procedures

All the commercially available materials were used without further purification otherwise noted. All reactions were monitored by thin-layer chromatography (TLC) on 0.25 mm silica gel plates (60 GF-254). The product spots were visualized by UV light, ferric chloride and iodine vapor. The purification of products was performed *via* column chromatography and recrystallization. Melting points were determined on an electrothermal melting point apparatus without correction. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were determined on a Bruker DRX spectrometer with TMS as an internal standard. Chemical shifts were described as  $\delta$  in parts per million and  $J$  in hertz. HRMS and ESI-MS were conducted by Shandong Analysis and Test Center.

**3.1.1. Preparation of (S)-methyl 4-methyl-2-(3-(prop-2-yn-1-yl)ureido)pentanoate (7).** Compound 6 (1.81 g, 10.00 mmol) was added to a mixture solution of DCM (40 mL) and saturated NaHCO<sub>3</sub> (40 mL). Then, triphosgene (0.98 g, 3.30 mmol) was added to the mixture gradually and stirred at 0 °C for 1.5 h. The organic layer was separated and dried over MgSO<sub>4</sub> for 15 min. After filtration, the filtrate was dropwise added into the solution of propargylamine (0.55 g, 10.00 mmol) and Et<sub>3</sub>N (1.21 g, 12.00 mmol) in anhydrous DCM (100 mL) at 0 °C. Subsequently, the mixture was stirred at 25 °C for 12 h. DCM was concentrated under vacuum and the residue was dissolved in EtOAc (100 mL). The mixture was washed with 10% HCl (3 × 100 mL), saturated NaHCO<sub>3</sub> (3 × 100 mL) and brine (3 × 100 mL), followed by drying over MgSO<sub>4</sub> overnight. After evaporation of EtOAc, the

Table 3 Half-life and intrinsic clearance of compound 3 in mouse liver microsomes

Compd	$T_{1/2}$ (min)	$CL_{int}(mic)$ ( $\mu$ L min <sup>-1</sup> mg <sup>-1</sup> )	$CL_{int}(liver)$ (mL min <sup>-1</sup> kg <sup>-1</sup> )	Remaining ( $T = 60$ min)	Remaining ( <sup>a</sup> NCF = 60 min)
3	94.9	14.6	57.8	69.4%	80.1%
Testosterone	5.5	252.1	998.5	0.2	91.0%

<sup>a</sup> NCF: abbreviation of no co-factor.





Table 4 The pharmacokinetic parameters of compound **3** after oral or intravenous administration in rats ( $n = 3$ )

PK parameters	Dosed (mg kg <sup>-1</sup> )	C <sub>max</sub> (ng mL <sup>-1</sup> )	T <sub>max</sub> (h)	AUC <sub>0–t</sub> (ng h mL <sup>-1</sup> )	AUC <sub>0–∞</sub> (ng h mL <sup>-1</sup> )	CL (mL h <sup>-1</sup> kg <sup>-1</sup> )	T <sub>1/2</sub> (h)	F (%)
i.v.	10	47 400 ± 15 838	0.0833 ± 0	16 059 ± 6837	16 067 ± 6840	737 ± 407	0.867 ± 0.387	NA
p.o.	50	337 ± 158	0.417 ± 0.144	484 ± 119	510 ± 99.3	NA	4.44 ± 4.1	0.64

obtained crude product was further purified by recrystallization from ethyl acetate/petroleum ether to give compound **7** as a white solid (1.87 g, yield: 83%), mp: 102.9–104.2 °C. <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ 6.37 (d, *J* = 8.4 Hz, 1H), 6.25 (t, *J* = 5.7 Hz, 1H), 4.17 (q, *J* = 8.4 Hz, 1H), 3.78 (dd, *J* = 5.7 Hz, *J* = 2.4 Hz, 2H), 3.61 (s, 3H), 3.05 (t, *J* = 2.4 Hz, 1H), 1.68–1.54 (m, 1H), 1.47–1.42 (m, 2H), 0.89–0.84 (m, 6H); ESI-MS *m/z* 227.4 [M + H]<sup>+</sup>.

**3.1.2. Preparation of (S)-N-hydroxy-4-methyl-2-(3-(prop-2-yn-1-yl)ureido)pentanamide (8).** Potassium hydroxide (28.50 g, 508.93 mmol) was dissolved in anhydrous methanol (70 mL) under an ice-bath. Then the above solution was added dropwise to hydroxylamine hydrochloride (23.00 g, 330.94 mmol) in the anhydrous methanol solution (120 mL) at 0 °C. The mixture was stirred at 0 °C for 40 min and the precipitate was filtered out to give a fresh methanol solution of potassium hydroxylamine. Compound **7** (2.26 g, 10 mmol) was dissolved in the solution above (20 mL) at 25 °C, followed by stirring at 25 °C for 0.5 h. The methanol was evaporated and the residue was dissolved in water. The excess base was neutralized with 10% HCl. Then, the formed oil was extracted by EtOAc (3 × 100 mL). The organic layer was washed with saturated NaHCO<sub>3</sub> (3 × 100 mL) and brine (3 × 100 mL), and dried over MgSO<sub>4</sub>. EtOAc was evaporated under vacuum, and the residue was added into DCM. The mixture was placed into refrigerator overnight. The white precipitate was formed and filtered off to give 1.20 g of compound **8**. Yield: 53%, mp: 124.5–126.2 °C. <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>): δ 10.68 (s, 1H), 8.80 (s, 1H), 6.24 (t, *J* = 5.7 Hz, 1H), 6.16 (d, *J* = 8.4 Hz, 1H), 4.05 (q, *J* = 8.4 Hz, 1H), 3.78 (dd, *J* = 5.7 Hz, *J* = 2.4 Hz, 2H), 3.05 (t, *J* = 2.4 Hz, 1H), 1.54–1.50 (m, 1H), 1.34–1.32 (m, 2H), 0.89–0.84 (m, 6H); ESI-MS *m/z* 226.3 [M-H]<sup>-</sup>.

**3.1.3. Preparation of methyl 2-azidobenzoate (10).** Compound **9** (12.08 g, 80.00 mmol) was dissolved in the 10% HCl (400 mL), and then the solution of NaNO<sub>2</sub> (5.91 g, 85.60 mmol) in H<sub>2</sub>O (50 mL) was added, the mixture was stirred at 0 °C for 0.5 h. Then, the solution of NaN<sub>3</sub> (5.72 g, 88.00 mmol) in H<sub>2</sub>O (50 mL) was dropwise added into the above mixture. After stirring at 0 °C for 0.5 h, the aqueous layer was extracted by EtOAc (3 × 150 mL). The organic layer was dried over MgSO<sub>4</sub> overnight. After filtration, EtOAc was evaporated to give the crude product, which was further purified by column chromatography (the fluent phase: petroleum ether) to give compound **10** as yellow oil (12.32 g, yield 87%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 7.77 (d, *J* = 8.0 Hz, 1H), 7.62 (t, *J* = 8.0 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.27 (t, *J* = 8.0 Hz, 1H), 3.83 (s, 3H).

**3.1.4. Preparation of 2-azidobenzoic acid (11).** To the solution of compound **10** (8.85 g, 50.00 mmol) in the MeOH (60

mL), the 1 N NaOH aqueous solution (60 mL) was added. After stirring at 25 °C for 3 h, 10% HCl was added to adjust pH to 6. The formed precipitate was filtered off and dried under vacuum to give compound **11** as a white solid (7.99 g, yield: 98%). mp: 140.5–141.4 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 13.17 (s, 1H), 7.78 (d, *J* = 8.0 Hz, 1H), 7.60 (t, *J* = 8.0 Hz, 1H), 7.36 (d, *J* = 8.0 Hz, 1H), 7.27 (t, *J* = 8.0 Hz, 1H).

**3.1.5. Preparation of 5-fluoro-1-(hydroxymethyl)pyrimidine-2,4(1*H*,3*H*)-dione (4).** Compound **1** (2.20 g, 16.90 mmol) was dissolved in 37% oxymethylene solution (3.01 g, 37.1 mmol) and the mixture was stirred at 70 °C for 2 h. The solvent was evaporated and the residue was dried under vacuum to give compound **4** as colorless oil. The crude product was used in the next reaction without further purification.

**3.1.6. Preparation of (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)methyl 2-azidobenzoate (12).** PyBOP (11.23 g, 21.60 mmol) and Et<sub>3</sub>N (2.18 g, 21.60 mmol) was added to a solution of compounds **11** (2.93 g, 17.98 mmol) and **4** (2.88 g, 18.00 mmol) in anhydrous DCM (20 mL) and anhydrous THF (20 mL). After stirring at 25 °C for 24 h, the solvent was evaporated and the residue was dissolved in EtOAc (50 mL) and H<sub>2</sub>O (50 mL). The formed precipitate was filtered out. The organic layer was separated and washed with brine. After dried over MgSO<sub>4</sub> overnight, EtOAc was evaporated to give the crude product, which was further purified by column chromatography (DCM/MeOH/THF = 100 : 2 : 40) to give compound **12** as a white solid (2.25 g, yield: 41%). mp: 168.4–169.8 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 12.03 (s, 1H), 8.26 (d, *J* = 6.5 Hz, 1H), 7.82 (d, *J* = 7.8 Hz, 1H), 7.68 (t, *J* = 7.8 Hz, 1H), 7.44 (d, *J* = 8.1 Hz, 1H), 7.31 (t, *J* = 7.8 Hz, 1H), 5.80 (s, 2H).

**3.1.7. Preparation of (5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)methyl (S)-2-(4-((3-(1-(hydroxyamino)-4-methyl-1-oxopentan-2-yl)ureido)methyl)-1*H*-1,2,3-triazol-1-yl)benzoate (3).** Compounds **8** (0.53 g, 2.33 mmol) and **12** (0.78 g, 2.60 mmol) were dissolved in DMSO (16 mL), followed by the addition of sodium ascorbate (68.3 mg, 0.35 mmol) in H<sub>2</sub>O (2 mL) and CuSO<sub>4</sub>·5H<sub>2</sub>O (30 mg, 0.12 mmol) in H<sub>2</sub>O (2 mL). After stirring at 25 °C for 5 h, the mixture was poured into water (100 mL). Then, the mixture was extracted by EtOAc (3 × 100 mL). The organic layer was washed with brine (3 × 100 mL) and dried over MgSO<sub>4</sub>. EtOAc was evaporated and the residue was purified by column chromatography (DCM/MeOH = 100 : 10) to give compound **3** as a white solid (0.31 g, yield: 25%). mp: 124.3–125.8 °C. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 11.97 (s, 1H), 10.68 (s, 1H), 8.80 (s, 1H), 8.31 (s, 1H), 8.00–7.95 (m, 2H), 7.83 (t, *J* = 7.7 Hz, 1H), 7.72 (t, *J* = 7.7 Hz, 1H), 7.65 (d, *J* = 7.7 Hz, 1H), 6.44 (t, *J* = 5.7 Hz, 1H), 6.21 (d, *J* = 8.8 Hz, 1H), 5.63 (s, 2H), 4.35–4.24 (m, 2H), 4.11–4.05 (m, 1H), 1.58–1.50 (m, 1H), 1.35 (t, *J* = 7.2 Hz,



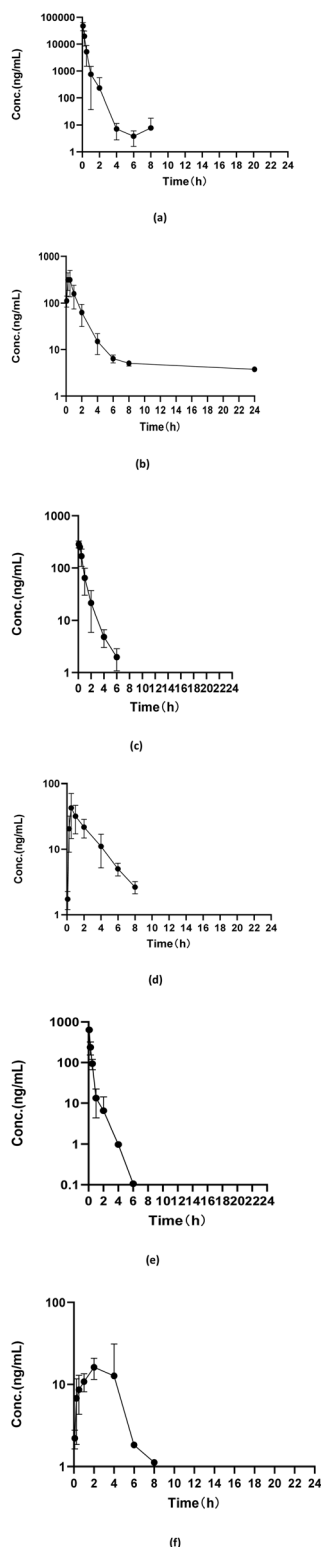


Fig. 5 (a) Concentration–time curve of compound 3 after i.v. administration in rats; (b) concentration–time curve of compound 3 after p.o. administration in rats; (c) concentration–time curve of compound 5 after i.v. administration in rats; (d) concentration–time curve of compound 5 after p.o. administration in rats; (e) concentration–time curve of compound 5-FU after i.v. administration in rats; (f) concentration–time curve of compound 5-FU after p.o. administration in rats.

2H), 0.89–0.85 (m, 6H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  169.9, 165.1, 157.7, 149.5, 146.7, 141.1, 138.8, 135.7, 133.9, 131.2, 130.3, 129.7, 129.4, 126.5, 124.1, 71.3, 49.6, 42.8, 35.2, 24.6, 23.2, 22.6. Retention time: 36.03 min, eluted with MeOH/water (0.1% formic acid), purity: 97.16%.

### 3.2. Biological evaluation

**3.2.1. APN inhibition assay.**  $\text{IC}_{50}$  values of tested compounds against APN were evaluated using L-Leu-p-nitro-anilide (Sigma-Aldrich, St. Louis, MO, USA) as substrate and microsomal APN from porcine kidney microsomes (Enzo Life Sciences, Farmingdale, NY, USA) as enzyme. The substrate was dissolved in DMSO for the concentration of  $16 \text{ mmol L}^{-1}$  and the enzyme was dissolved in 50 mM PBS ( $\text{pH} = 7.2$ ) to a concentration of  $0.15 \text{ IU L}^{-1}$ . The tested compounds were dissolved in DMSO for the concentration of  $100 \text{ mmol L}^{-1}$  as stock solutions, which were further diluted to the concentrations of  $250 \mu\text{mol L}^{-1}$ ,  $62.5 \mu\text{mol L}^{-1}$ ,  $15.63 \mu\text{mol L}^{-1}$ ,  $3.91 \mu\text{mol L}^{-1}$ ,  $0.98 \mu\text{mol L}^{-1}$ , and  $0.24 \mu\text{mol L}^{-1}$  using PBS buffer, respectively. Briefly, compounds ( $40 \mu\text{L}$ ) were added into 96-well plates, followed by the addition of PBS ( $145 \mu\text{L}$ ). Then, substrate ( $5 \mu\text{L}$ ) and enzyme ( $10 \mu\text{L}$ ) were added into the above solution in sequence. The mixture was incubated at  $37^\circ\text{C}$  for 30 min. The absorbance values were measured at 405 nm with a plate reader (Varioskan, Thermo Fisher Scientific, Waltham, MA, USA).

**3.2.2. Anti-proliferation assay.** Anti-proliferative activities of compounds against tumor cells were evaluated by the MTT [(3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide)] method. Before the experiment, the tumor cells were taken out of the liquid nitrogen tank and resuscitated in a  $40^\circ\text{C}$  water bath for 1 minute. Firstly, the resuscitated tumor cells were cultivated in RPMI 1640 medium with 10% FBS at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  humidified incubator. The MTT test was conducted when the tumor cells were in the logarithmic growth phase. Cells suspended in culture medium ( $100 \mu\text{L}$ ) were inoculated in 96-well plates with the density of 10 000–12 000 cells per well. After incubation for 4 h, compound sample ( $100 \mu\text{L}$ ) which were previously diluted to the working concentrations ( $0.16/0.8/4/20/100 \mu\text{mol L}^{-1}$ ) with complete medium were added to the tested 96-well plates and incubated for a further 48 h. Subsequently, MTT ( $20 \mu\text{L}$ ,  $5 \text{ mg mL}^{-1}$ ) solution was added into the test well, followed by the incubation for another 4 h. The plates were centrifuged at 800 rpm for 3 min. The supernatant was poured off and DMSO ( $200 \mu\text{L}$ ) was added to dissolve the formed formazan. Finally, the mixture was shaken for 15 min and the absorbance values were measured using a plate reader at 570 nm (Varioskan, Thermo Fisher Scientific, Waltham, MA, USA).

**3.2.3. HUVEC tubular structure formation assay.** Matrigel ( $50 \mu\text{L}$ ; BD biosciences) was added into test well of 96-well plates and then allowed to polymerize for 0.5 h at  $37^\circ\text{C}$ . HUVECs were suspended in M199 medium at a density of  $4 \times 10^5$  cells per mL.  $50 \mu\text{L}$  of cell suspension was added into the well pre-coated with Matrigel, followed by the addition of  $50 \mu\text{L}$  of compounds dissolved in M199 medium at the specified concentrations. The mixture was further incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 4 h. The



tubular structures formed by HUVECs were observed under an inverted microscope and photographed at 100× magnification.

**3.2.4. Rat aortic ring assay.** The thoracic aortas were separated from 8 to 10 week-old male Sprague Dawley rats. After careful removal of fibroadipose tissues, the aortas were cut into 1 mm-long cross-section. The test wells of the 96-well plates were pre-coated with Matrigel (100  $\mu$ L; BD bioscience) at 37 °C for 0.5 h, followed by the addition of the aortas fragments and 100  $\mu$ L of Matrigel, successively. The mixture was incubated at 37 °C and 5% CO<sub>2</sub> for 0.5 h until the Matrigel solidified. Compounds dissolved in M199 culture medium at the test concentrations were added into the above wells, followed by the incubation at 37 °C in 5% CO<sub>2</sub> for 9 days. The formed microvessels were visualized by inverted microscope at 100× magnification. The M199 culture medium containing compounds was changed every three days.

**3.2.5. Stability of the test compound *in vitro*.** Simulated gastric fluid was prepared as follows. Dissolved 0.04 g NaCl and 0.064 g pepsin in 0.14 mL HCl and added sufficient H<sub>2</sub>O to make the total volume of 20 mL. The pH value of the test solution was determined as 1.20  $\pm$  0.05 by pH meter.

Simulated intestinal fluid was prepared as follows. Dissolved 0.136 g KH<sub>2</sub>PO<sub>4</sub> and 0.2 g pancreatin and added sufficient H<sub>2</sub>O to make the total volume of 20 mL. The pH value of the test solution was adjusted to 6.8  $\pm$  0.05.

To the stock solution of compound 3 was added simulated gastric fluid and simulated intestinal fluid, of which the final test concentration was 2  $\mu$ M. The mixture was incubated at 37 °C and 600 rpm for the appointed time. After incubation of 60 min, 120 min, 360 min and 1440 min, respectively, to the test samples were added 400  $\mu$ L of cold acetonitrile containing 200 ng per mL tolbutamide and labetalol (internal standard) immediately. Then, 200  $\mu$ L of suspension was separated and mixed with 400  $\mu$ L of cold acetonitrile containing 200 ng per mL tolbutamide and labetalol again. Subsequently, samples were subjected to centrifuge at 4000 rpm, 4 °C for 20 min. 200  $\mu$ L of supernatant was used for the LC/MS/MS analysis to determine the remaining amount of test compound based on peak area ratio of analyte/IS. LC/MS/MS analysis was using a ACQUITY UPLC BEH C18 column (1.7  $\mu$ m 2.1  $\times$  50 mm). Compounds were eluted with water (containing 0.1% formic acid)/acetonitrile (containing 0.1% formic acid) over 60 min. The absorbance was measured at 282 nm, the flow rate was 0.5 mL min<sup>-1</sup> and the quantity of injection was 20  $\mu$ L.

**3.2.6. Metabolic stability in mouse liver microsomes (MLMs).** CD-1 mouse (Lot#: 2010017) liver microsomes obtained from Xenotech were used to determine the metabolic stability of compound 3. Firstly, NADPH (final concentration = 1 mM) (Lot#: 00616) and MLMs were individually added to potassium phosphate buffer (100 mM) and incubated at 37 °C for 10 min. Subsequently, compound 3 and the positive control testosterone were prepared with 5  $\mu$ L DMSO and 495  $\mu$ L acetonitrile (ACN), and then spiked into the reaction mixture above to achieve a final concentration of 1  $\mu$ M, respectively. Samples were separated from the reaction mixture at different times (5 min, 15 min, 30 min, 45 min, and 60 min), and the reaction was terminated using cold acetonitrile (ACN, 4 °C) containing 200 ng mL<sup>-1</sup> of tolbutamide and 200 ng mL<sup>-1</sup> of labetalol as internal standards (IS). Each

bioanalysis plate was sealed and shaken for 10 minutes prior to LC-MS/MS analysis. The equation of first order kinetics was used to calculate  $T_{1/2}$  and  $CL_{int}(mic)$  ( $\mu$ L min<sup>-1</sup> mg<sup>-1</sup>).

**3.2.7. *In vivo* PK studies.** The pharmacokinetic parameters of compound 3 were evaluated in male Sprague Dawley rats ( $N = 3$ ) after an intravenous or oral administration. The rats weighing 180–220 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (SCXK-2021-0011). All protocols involving animals were approved by the XBL-China Institutional Animal Care and Use Committee (IACUC no. 2022-0314). In order to adjust to the environment, the rats were supplied with water and a commercial rodent diet in a controlled room with relative humidity at 40–70% and temperature at 20–26 °C for one week prior to the study. However, the rats were fasted for 12 h before the experiment. Compound 3 was administrated intravenously at a dose of 10 mg kg<sup>-1</sup> or orally at a dose of 50 mg kg<sup>-1</sup>, respectively. Samples were collected at several time points up to 24 h following dose administration. Following intravenous infusion administration, blood samples were collected from the jugular vein cannula at predose as well as at 0.083 h, 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h postdose of compound 3. Moreover, following oral dosing, 0.25 mL of blood samples were collected at predose as well as at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h and 24 h postdose of compound 3. Rat plasma samples were separated from the blood samples by centrifugation at 6000 rpm for 3 min and stored in the freezer at –20 °C before analysis. Non-compartmental PK parameters such as area under the curve (AUC) and  $T_{1/2}$  were calculated using WinNonlin 8.2. The absolute oral bioavailability of compound 3 in rats was calculated by the AUC(0– $\infty$ ) ratio obtained following oral and i.v. administration.

$$F(\%) = (\text{dose}_{iv} \times \text{AUC}_{\text{oral}(0-\infty)}) / (\text{dose}_{\text{oral}} \times \text{AUC}_{iv(0-\infty)}) \times 100\%$$

## 4. Conclusions

In summary, according to the previous series of leucine ureido derivatives containing the 1,2,3-triazole moiety as APN inhibitors, we designed and synthesized the conjugated compound 3 following the multi-target drug design approach. Compound 3 exhibited more potent anti-proliferative activities against two human leukemic cell lines and anti-angiogenesis activity compared with the positive control bestatin. Furthermore, the preliminary stability of compound 3 revealed that the hybrid could release significant level of compound 5 and 5-FU *in vitro*, while NADPH was involved in the metabolism. Moreover, *in vivo* PK studies revealed that compound 3 was absorbed rapidly after oral administration, but the absolute oral bioavailability was approximately 0.64% in rats. Therefore, further attention can be focused on increasing the oral bioavailability and enhancing the pharmacological efficacy.

## Data availability

The data supporting this article have been included as part of the ESI.†



## Author contributions

Conceptualization, Jiangying Cao, Chunxi Liu and Anchang Liu; data curation, Fangyuan Shi, Yingjie Zhang and Qifu Xu; formal analysis, Fangyuan Shi, Yingjie Zhang and Qifu Xu; investigation, Fangyuan Shi and Qifu Xu; methodology, Jiangying Cao, Chunxi Liu and Anchang Liu; project administration, Jiangying Cao, Chunxi Liu and Anchang Liu; supervision, Jiangying Cao, Chunxi Liu and Anchang Liu; writing – original draft, Fangyuan Shi; writing – review & editing, Jiangying Cao, Chunxi Liu and Anchang Liu.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 82003578) and Shandong Provincial Natural Science Foundation, China (No. ZR2020QH341).

## Notes and references

- 1 N. D. Rawlings and A. J. Barrett, MEROPS: the peptidase database, *Nucleic Acids Res.*, 1999, **27**(1), 325–331.
- 2 C. Antczak, I. De Meester and B. Bauvois, Ecto-peptidases in pathophysiology, *Bioessays*, 2001, **23**(3), 251–260.
- 3 Y. Luan and W. Xu, The structure and main functions of aminopeptidase N, *Curr. Med. Chem.*, 2007, **14**(6), 639–647.
- 4 P. Mina-Osorio, The moonlighting enzyme CD13: old and new functions to target, *Trends Mol. Med.*, 2008, **14**(8), 361–371.
- 5 T. Tokuhara, N. Hattori, H. Ishida, T. Hirai, M. Higashiyama, K. Kodama and M. Miyake, Clinical significance of aminopeptidase N in non-small cell lung cancer, *Clin. Cancer Res.*, 2006, **12**(13), 3971–3978.
- 6 Y. Aozuka, K. Koizumi, Y. Saitoh, Y. Ueda, H. Sakurai and I. Saiki, Anti-tumor angiogenesis effect of aminopeptidase inhibitor bestatin against B16-BL6 melanoma cells orthotopically implanted into syngeneic mice, *Cancer Lett.*, 2004, **216**(1), 35–42.
- 7 L. Guzman-Rojas, R. Rangel, A. Salameh, J. K. Edwards, E. Dondossola, Y. G. Kim, A. Saghatelian, R. J. Giordano, M. G. Kolonin, F. I. Staquicini, *et al.*, Cooperative effects of aminopeptidase N (CD13) expressed by nonmalignant and cancer cells within the tumor microenvironment, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**(5), 1637–1642.
- 8 N. Haraguchi, H. Ishii, K. Mimori, F. Tanaka, M. Ohkuma, H. M. Kim, H. Akita, D. Takiuchi, H. Hatano, H. Nagano, *et al.*, CD13 is a therapeutic target in human liver cancer stem cells, *J. Clin. Invest.*, 2010, **120**(9), 3326–3339.
- 9 A. Mucha, M. Drag, J. P. Dalton and P. Kafarski, Metallo-aminopeptidase inhibitors, *Biochimie*, 2010, **92**(11), 1509–1529.
- 10 S. A. Amin, N. Adhikari and T. Jha, Design of Aminopeptidase N Inhibitors as Anti-cancer Agents, *J. Med. Chem.*, 2018, **61**(15), 6468–6490.
- 11 Y. Mishima, Y. Terui, N. Sugimura, Y. Matsumoto-Mishima, A. Rokudai, R. Kuniyoshi and K. Hatake, Continuous treatment of bestatin induces anti-angiogenic property in endothelial cells, *Cancer Sci.*, 2007, **98**(3), 364–372.
- 12 M. Lis, M. Szczypka, A. Suszko and B. Obminska-Mrukowicz, Influence of bestatin, an inhibitor of aminopeptidases, on T and B lymphocyte subsets in mice, *Pol. J. Vet. Sci.*, 2011, **14**(3), 393–403.
- 13 Z. Y. Tian, G. J. Du, S. Q. Xie, J. Zhao, W. Y. Gao and C. J. Wang, Synthesis and bioevaluation of 5-fluorouracil derivatives, *Molecules*, 2007, **12**(11), 2450–2457.
- 14 X. Pan, C. Wang, F. Wang, P. Li, Z. Hu, Y. Shan and J. Zhang, Development of 5-Fluorouracil derivatives as anticancer agents, *Curr. Med. Chem.*, 2011, **18**(29), 4538–4556.
- 15 N. Shimma, I. Umeda, M. Arasaki, C. Murasaki, K. Masubuchi, Y. Kohchi, M. Miwa, M. Ura, N. Sawada, H. Tahara, *et al.*, The design and synthesis of a new tumor-selective fluoropyrimidine carbamate, capecitabine, *Bioorg. Med. Chem.*, 2000, **8**(7), 1697–1706.
- 16 J. Cao, J. Zang, X. Kong, C. Zhao, T. Chen, Y. Ran, H. Dong, W. Xu and Y. Zhang, Leucine ureido derivatives as aminopeptidase N inhibitors using click chemistry. Part II, *Bioorg. Med. Chem.*, 2019, **27**(6), 978–990.
- 17 J. Cao, C. Ma, J. Zang, S. Gao, Q. Gao, X. Kong, Y. Yan, X. Liang, Q. Ding, C. Zhao, *et al.*, Novel leucine ureido derivatives as aminopeptidase N inhibitors using click chemistry, *Bioorg. Med. Chem.*, 2018, **26**(12), 3145–3157.
- 18 Z. Chen, L. Han, M. Xu, Y. Xu and X. Qian, Rationally designed multitarget anticancer agents, *Curr. Med. Chem.*, 2013, **20**(13), 1694–1714.
- 19 L. M. Espinoza-Fonseca, The benefits of the multi-target approach in drug design and discovery, *Bioorg. Med. Chem.*, 2006, **14**(4), 896–897.

