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Design, synthesis and biological evaluation of novel non-covalent piperidine-containing peptidyl proteasome inhibitors

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Abstract: A series of novel non-covalent piperidine-containing dipeptidyl derivatives were designed, synthesized and evaluated as proteasome inhibitors. All target compounds were tested for their proteasome chymotrypsin-like inhibitory activities, and selected derivatives were evaluated for the anti-proliferation activities against two multiple myeloma (MM) cell lines RPMI 8226 and MM-1S. Among all of these compounds, eight exhibited significant proteasome inhibitory activities with IC_{50} less than 20 nM, and four are more potent than the positive control Carfilzomib. Compound **28** displayed the most potent proteasome inhibitory activity (IC_{50}: 1.4±0.1 nM) and cytotoxicities with IC_{50} values at 13.9±1.8 nM and 9.5±0.5 nM against RPMI 8226 and MM-1S, respectively. Additionally, the *ex vivo* blood cell proteasome inhibitory activities of compounds **24** and **27-29** demonstrated that the enzymatic metabolism in the whole blood could be well tolerated. All these experiments confirmed that the piperidine-containing non-covalent proteasome inhibitors are potential leads for exploring new anti-cancer drugs.

Keywords: Proteasome inhibitors; Piperidine; Non-covalent; Anti-cancer; SARs

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1. Introduction

The ubiquitin-proteasome pathway (UPP), which regulates various critical mediators of different signaling pathways, is essential for maintaining normal cell function and cellular homeostasis.[1-4] This proteolytic pathway includes several components: ubiquitin, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), ubiquitin protein ligase (E3), deubiquitinating enzyme (DUB) and the dominant proteasome, most of which have been proved to be potential drug targets.[5-8] The 26S proteasome, a large protein complex with multiple proteolytic activities, is consisted of a 4 stacked ring-formed 20S cylindrical core (α7-β7-β7-α7) and two 19S regulatory particles.[9-10] Three β subunits (β1, β2 and β5) exhibit caspase-like (C-L), trypsin-like (T-L) and chymotrypsin-like (CT-L) activities, respectively.[10] Among all of these potential targets, the validated anti-cancer drug target proteasome has played critical roles in discovery of multiple myeloma (MM) therapy drugs.[11-12] To date, three proteasome inhibitors Bortezomib, Carfilzomib and Ixazomib (Fig. **1**) have been approved for the treatment of MM.[13-15] Besides, several other proteasome inhibitors are being extensively evaluated in various clinical trials.[10]

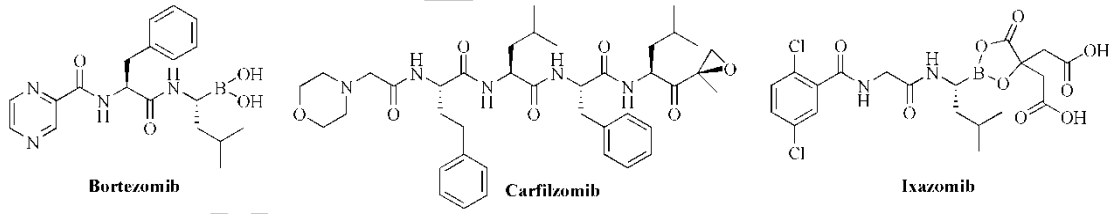


Figure 1. Structures of the three approved proteasome inhibitors

Proteasome inhibitors can be classified into covalent and non-covalent types due to different structure scaffolds and binding modes with proteasome.[16] Most proteasome inhibitors approved or in clinical trials are covalent ones. The firm covalent interactions ensure these small molecular ligands with potent and lasting proteasome inhibitory activities, but may induce severe side effects and limit their tissue distribution for lack of specificity and excessively reactive.[17-19] Non-covalent proteasome inhibitors may offer more therapeutic advantages due to their more widespread tissue distribution compared to the covalent inhibitors.[19-20] Although non-covalent proteasome inhibitors are less well studied, the history of these analogues is as long as that of the covalent inhibitors. CVT-659 (Fig. **2**) is the forerunner of this kind of inhibitors with an IC_{50} of 140 nM.[21] Additionally, a trimethoxy-L-phenylalanine-containing dipeptide (**2**, Fig. **2**) was reported with potent and selective chymotrypsin-like activity together with moderate cytotoxicity.[22] Besides, Blackburn and colleagues

described a series of di- and tripeptides (e.g., **3** and **4**, Fig. **2**) with both potent constitutive proteasome and immunoproteasome inhibitory activities.[19]

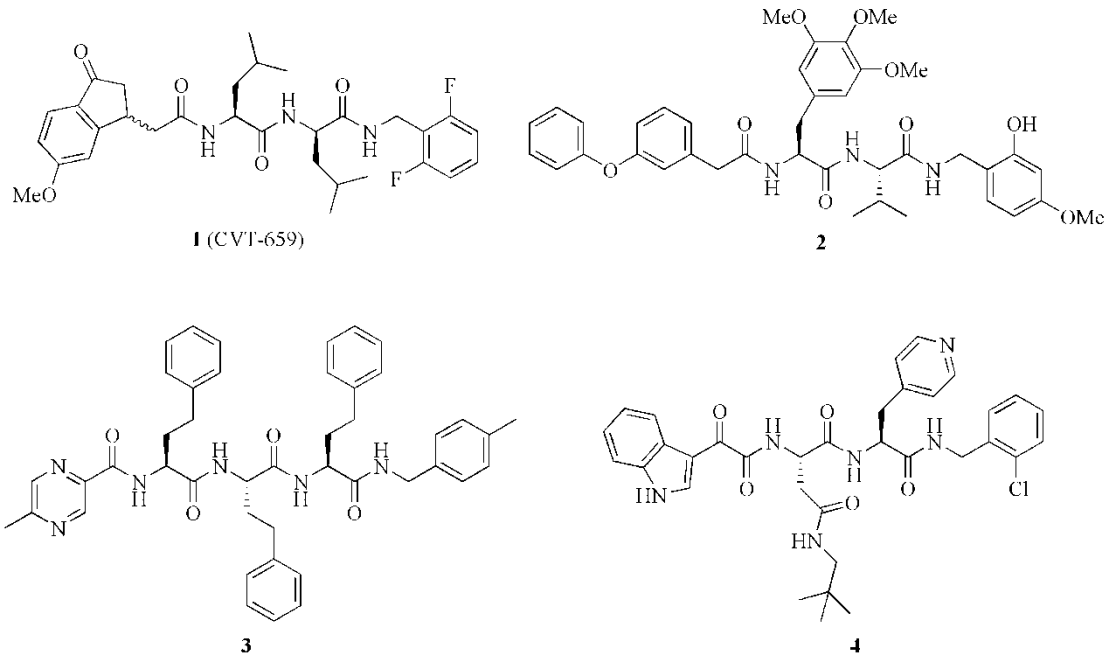


Figure 2. Structures of representative non-covalent proteasome inhibitors

Since most of the reported non-covalent proteasome inhibitors are short peptides, the enzymatic stability of these compounds should be well concerned.[23] Introducing a non-peptide fragment into the peptide skeleton may solve this problem and increase the pharmacokinetic properties of the target compounds. This is mainly owing to the specificity of proteases and peptidases against peptide bond between peptide fragments. In this manuscript, a series of piperidine-containing non-covalent proteasome inhibitors (Fig. **3**) were synthesized and evaluated, and structure-activity relationships (SARs) were discussed in detail.

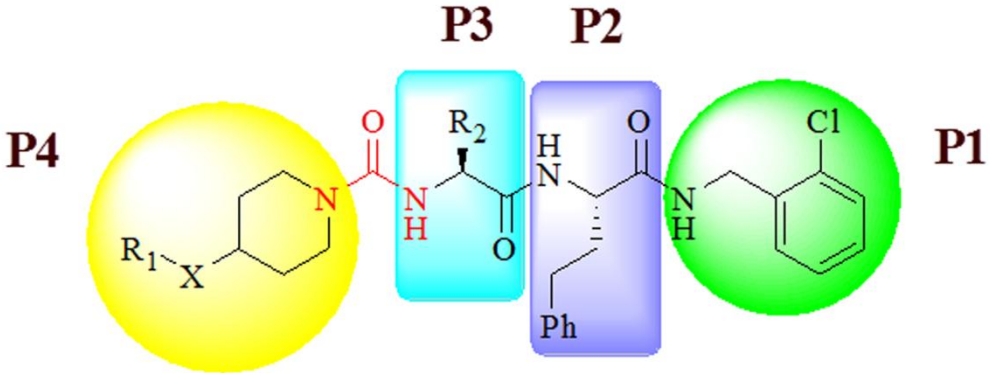


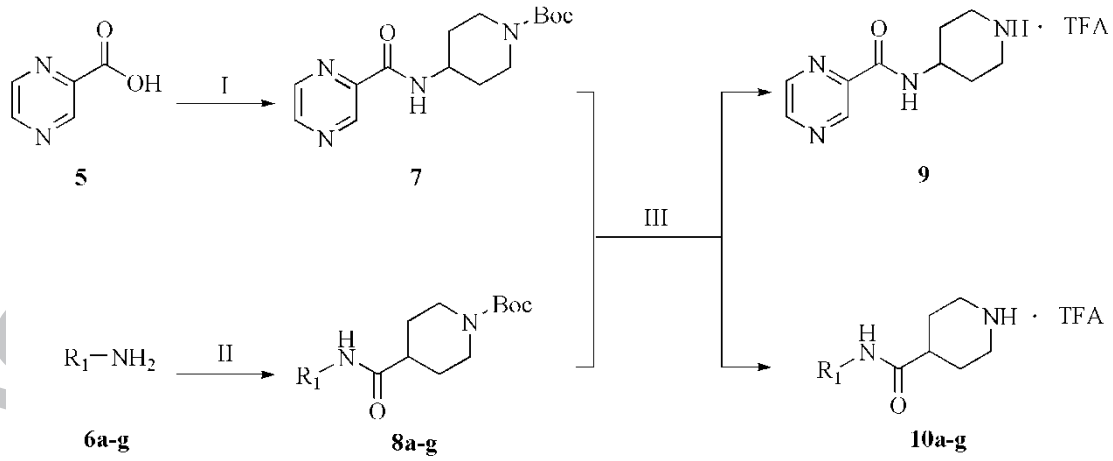
Figure 3. Non-peptide fragment (piperidine) constructed non-covalent proteasome inhibitors

2. Results and discussion

2.1 Chemistry

The synthetic route for piperidine-containing fragments **9** and **10a-g** are summarized in Scheme **1**. Fragment **7** can be easily obtained by condensation from pyrazine-2-carboxylic acid with 1-Boc-4-aminopiperidine. However, synthesis of the similar arylcarbamoyl piperidine derivatives **8a-g** were more difficult, in which the *N*-Boc-4-piperidinecarboxylic acid should first be transformed to the acyl chloride at the presence of $SOCl_2$, then reacted with corresponding arylamine **6a-g** to afford the products.[24] Afterwards, deprotection of **7** and **8a-g** with trifluoroacetic acid (TFA) resulted in piperidine TFA salts **9** and **10a-g**.

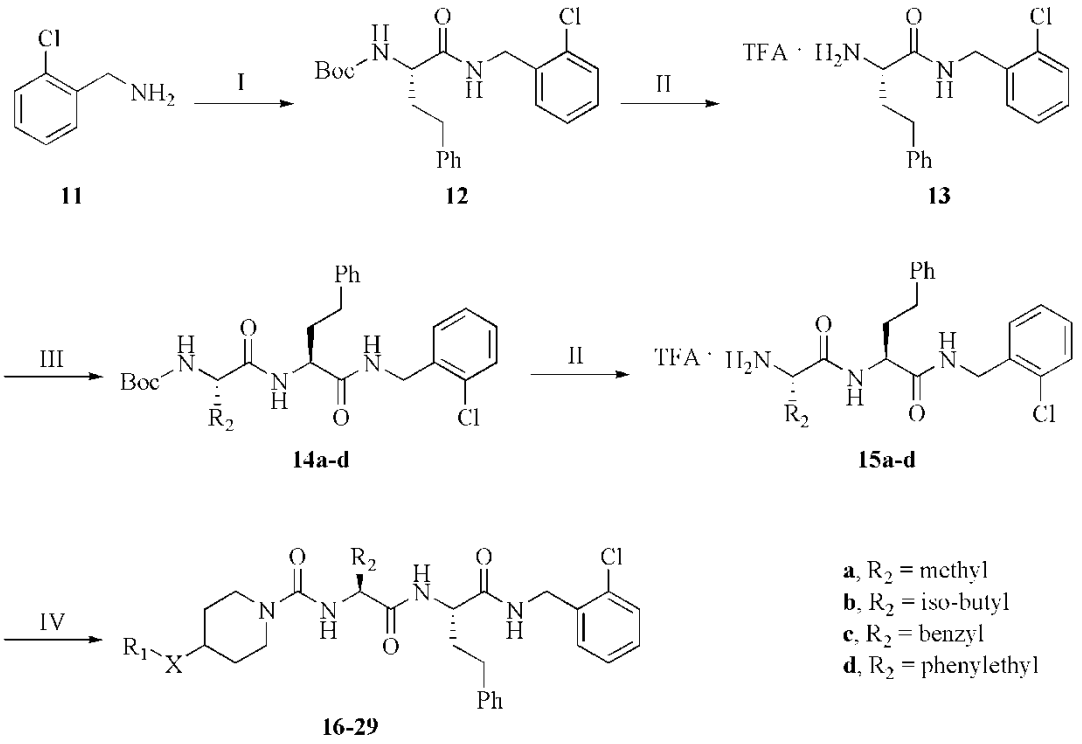
The target compounds **16-29** were synthesized following the method described in Scheme **2**. Reaction of (2-chlorophenyl)methanamine with Boc-L-hPhe furnished compound **12**, which was deprotected, treated with various Boc-protected amino acid and deprotected again to afford dipeptide TFA salts **15a-d**. Subsequently, the primary amine **15a-d** were first transformed to corresponding isocyanate intermediates, which were not stable enough and were thereby reacted with piperidine fragments **9** and **10a-g** to obtain target compounds **16-29**.[25]

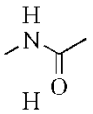
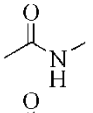
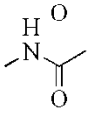
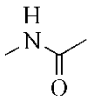
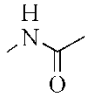
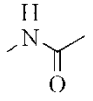


a, R_1= pyrazin-2-yl b, R_1= phenyl c, R_1= 4-fluorophenyl d, R_1= 4-methoxyphenyl

e, R_1= pyridin-2-yl f, R_1= pyridin-3-yl g, R_1= thiazol-2-yl

Scheme 1. Synthesis of piperidine-containing fragments (**9** and **10a-g**). Reagents and conditions: (I) HOBt, EDCI, 1-Boc-4-aminopiperidine, diisopropylethylamine, DCM, 0°C-rt; (II) *N*-Boc-4-piperidinecarboxylic acid, $SOCl_2$, pyridine, Et_3N, DMAP, DCM, rt; (III) trifluoroacetic acid, DCM, 0°C-rt.



	R_1	X	R_2		R_1	X	R_2
16	pyrazin-2-yl		methyl	**17**	pyrazin-2-yl		methyl
18	pyrazin-2-yl		iso-butyl	**19**	pyrazin-2-yl		iso-butyl
20	pyrazin-2-yl		benzyl	**21**	pyrazin-2-yl		benzyl
22	pyrazin-2-yl		phenylethyl	**23**	pyrazin-2-yl		phenylethyl
24	phenyl		phenylethyl	**25**	4-fluorophenyl		phenylethyl
26	4-methoxyphenyl		phenylethyl	**27**	pyridin-2-yl		phenylethyl
28	pyridin-3-yl		phenylethyl	**29**	thiazol-2-yl		phenylethyl

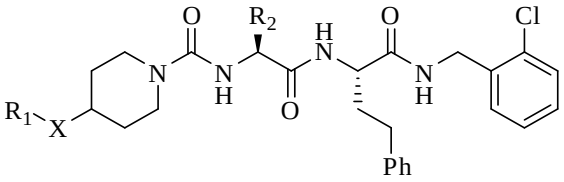
Scheme 2. Synthesis of piperidine-containing target compounds (**16-29**). Reagents and conditions: (I) Boc-L-hPhe, HOBt, EDCI, diisopropylethylamine, DCM, 0°C-rt; (II) trifluoroacetic acid, DCM, 0°C-rt; (III) corresponding Boc-protected amino acid, HOBt, EDCI, diisopropylethylamine, DCM, 0°C-rt; (IV) triphosgene, diisopropylethylamine, DCM, aqueous $NaHCO_3$, 0°C-rt.

2.2 Proteasome inhibitory activities

The synthesized target compounds were evaluated for their 20S proteasome chymotrypsin-like inhibitory activities *in vitro*. Carfilzomib was employed as the positive control. The results are summarized in Table **1**.

As illustrated in Table **1**, most target compounds showed potent proteasome inhibitory activities with IC_{50} lower than 100 nM, and 4 compounds were even lower than 10 nM, which indicated that the activities of these compounds were well maintained after introducing the piperidine ring into the peptide skeleton. Different substituents at P2 position (R_2) influenced the activity obviously. Iso-butyl and phenylethyl substituted analogues (**18**, **19**, **22** and **23**) exhibited much more potent activities than methyl and benzyl substituted compounds (**16**, **17**, **20** and **21**), with IC_{50} values of 16.6±1.7 nM, 45.7±1.1 nM, 14.7±2.4 nM and 23.7±3.5 nM, respectively. Phenylethyl showed the superiority and was selected at the P2 position for further optimization. The linker between the piperidine ring and the *N*-Cap (R_1) is also essential for the inhibitory activity. Compounds with carbamoyl group (NHCO) at X position were more potent than acylamino group (CONH) substituted compounds (**16** versus **17**; **18** versus **19**; **20** versus **21**; **22** versus **23**). Additionally, the structure-activity relationship at the *N*-Cap (R_1) suggested that replacements at this position had little influence on their activities. Generally speaking, replacements at R_1 position displayed little impact for the proteasome inhibitory activities of this series of compounds.

Table **1**. 20S proteasome chymotrypsin-like inhibitory activities of target compounds (**16-29**)



16-29

Compound	R_1	X	R_2	IC_{50} (nM)[a]
16	pyrazin-2-yl	-NHCO-	methyl	601.4±129.0
17	pyrazin-2-yl	-CONH-	methyl	669.9±94.9
18	pyrazin-2-yl	-NHCO-	iso-butyl	16.6±1.7
19	pyrazin-2-yl	-CONH-	iso-butyl	45.7±1.1
20	pyrazin-2-yl	-NHCO-	benzyl	152.9±26.3

21	pyrazin-2-yl	-CONH-	benzyl	343.9±69.7
22	pyrazin-2-yl	-NHCO-	phenylethyl	14.7±2.4
23	pyrazin-2-yl	-CONH-	phenylethyl	23.7±3.5
24	phenyl	-NHCO-	phenylethyl	3.0±0.4
25	4-fluorophenyl	-NHCO-	phenylethyl	16.9±2.0
26	4-methoxyphenyl	-NHCO-	phenylethyl	15.7±1.3
27	pyridin-2-yl	-NHCO-	phenylethyl	2.4±1.0
28	pyridin-3-yl	-NHCO-	phenylethyl	1.4±0.1
29	thiazol-2-yl	-NHCO-	phenylethyl	4.3±0.9
Carfilzomib	-	-	-	8.4±0.9

[a] The IC_{50} values are an average of three independent determinations.

2.3 Tumor cell growth inhibitory activities

Based on the proteasome chymotrypsin-like inhibitory activities, selected compounds (**18**, **22** and **24-29**) were further tested for their cytotoxic activities *in vitro* against two MM cell lines (RPMI 8226 and MM-1S) by MTS assay with Carfilzomib employed as the positive control. According to the results are summarized in Table **2**, 6 compounds exhibited potent anti-proliferation activities against two MM cell lines with IC_{50} values in low nanomolar range, which was consistent with the proteasome inhibitory activities. Among all of these compounds, **28** displayed the most potent cytotoxic activities with IC_{50} of 13.9±1.8 nM and 9.5±0.5 nM against RPMI 8226 and MM-1S cell line, respectively.

Table **2**. Cytotoxic activities of selected compounds (**18**, **22** and **24-29**) against two MM cell lines

Compound	Cytotoxicity (IC_{50}, nM) [a]	
	RPMI 8226	MM-1S
18	371.1±23.4	260.9±9.7
22	195.7±18.1	135.3±6.6
24	18.5±2.9	20.2±1.4
25	94.3±3.4	106.7±5.7
26	84.6±5.9	92.9±6.1
27	24.7±1.3	22.2±3.3

28	13.9±1.8	9.5±0.5
29	29.5±1.1	32.5±3.4
Carfilzomib	13.2±0.6	1.5±0.6

[a] The IC_{50} values are an average of three independent determinations.

2.4 *Ex vivo* blood cell proteasome inhibitory activities

Subsequently, four compounds (**24** and **27-29**) were further evaluated for the blood cell proteasome inhibitory activities *ex vivo*, and Carfilzomib was used as the positive control. As illustrated in Fig. **4**, **28** displayed the most potent blood cell proteasome inhibitory activities with inhibitory rates of more than 60% at all the three concentrations (0.5 μM, 2.5μM and 12.5μM), which are comparable to that of the Carfilzomib and is consistent with the *in vitro* proteasome inhibitory activity and cytotoxicities. The impressive *ex vivo* activities suggested that the stability of this series of compounds were improved by constructing a piperidine ring into the peptide skeleton, which was validated by the potent activities after metabolism by various enzymes in the whole blood.

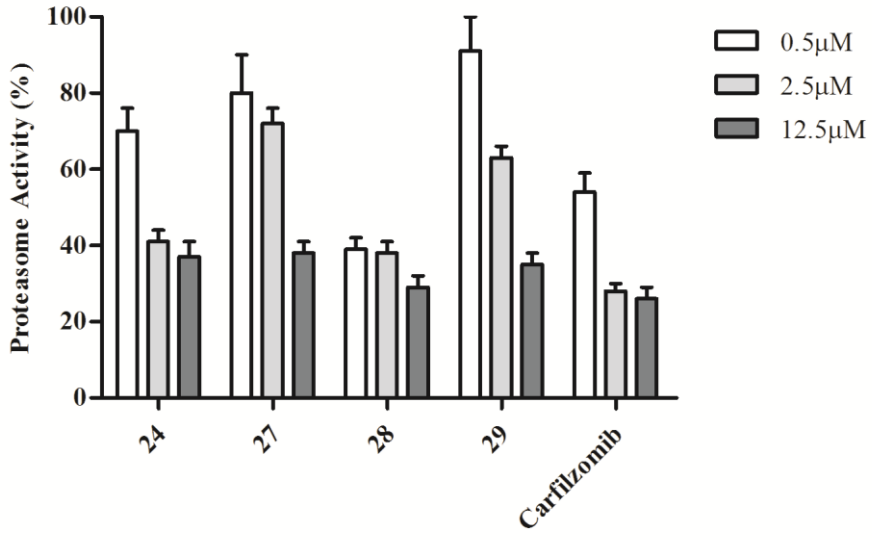


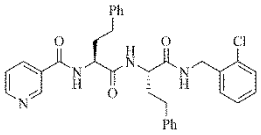
Figure 4. *Ex vivo* blood cell proteasome inhibitory activities of selected compounds (**24** and **27-29**)

2.5 Plasma and blood stability

The most potent compound **28** and its corresponding dipeptidyl analogue without piperidine ring (3-pyridinyl-carbonyl-hPhe-hPhe-2-chlorobenzylamide) were further tested for the stability by enzymatic degradation in mouse plasma and blood. As illustrated in Table **3**, compound **28** showed a

longer half life both in plasma and blood (435.3 min and 178.7 min, respectively) compared to the compound without a piperidine ring (285.3 min and 111.0 min, respectively), which demonstrated the availability of piperidine ring in increasing the enzymatic stability of this series of compounds.

Table **3**. Enzymatic stability of compound **28** and its corresponding dipeptidyl analogue without piperidine ring

Compound	Half-life ($T_{1/2}$, min)	
	plasma	blood
28	435.3	178.7
	285.3	111.0

3. Conclusion

A series of novel piperidine-containing non-covalent dipeptidyl derivatives were synthesized and evaluated for their proteasome inhibitory activities. Among these compounds, ten exhibited forceful proteasome inhibitory activities together with anti-proliferation activities against two MM cell lines RPMI 8226 and MM-1S, and these results are consistent with each other. In addition, compound **28** showed comparable *ex vivo* activity to the lead compound Carfilzomib with enhanced enzymatic stability, which both demonstrated the validity of the design concept. Besides, the linker between piperidine ring and the *N*-Cap group is also essential for the activity, which may be caused by different hydrogen bond interaction with the proteasome. This study validated the potential of developing non-peptide fragment constructed peptides as non-covalent proteasome inhibitors, which will be helpful for searching new MM therapy drugs.

4. Experimental procedures

4.1 Chemistry

^{1}H and ^{13}C NMR spectra were recorded on Brüker 500/400MHz spectrometer (Brüker Bioscience, Billerica, MA, USA) with $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts (δ) were reported in parts per million (ppm) relative to internal TMS, and coupling constants (*J*) were reported in Hertz (Hz). Splitting patterns were designated as singlet (s), broad singlet (brs), doublet (d), double doublet (dd), triplet (t), quartet (q) and multiplet (m). Mass spectral data were obtained by Esquire-LC-00075

spectrometer (Brüker Bioscience). Reagents and solvents were purchased from common commercial suppliers and were used without further purification unless stated otherwise. Column chromatography was performed using silica gel (300-400 mesh). All yields are unoptimized and generally represent the result of a single experiment.

4.1.1 **tert-Butyl 4-(Pyrazine-2-carboxamido) piperidine-1-carboxylate (7)**

To a suspension of pyrazine-2-carboxylic acid (**5**, 1.24g, 10.0mmol) in DCM (40.0mL), HOBt (1.49g, 11.0mmol) and EDCI (2.88g, 15.0mmol) were added at 0°C. The reaction mixture was kept at 0°C and was stirred for 30min. Then 1-Boc-4-aminopiperidine (2.00g, 10.0mmol) and diisopropylethylamine (3.7mL, 20.0mmol) were added. After stirring at room temperature for 3h, the resulting mixture was washed with aqueous $NaHCO_3$ solution (1×30mL), brine (1×30mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (ethyl acetate: petroleum ether = 1:1).

White solid; Yield: 95%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 9.40 (s, 1H, pyrazine-H), 8.75 (d, 1H, J = 2.0 Hz, pyrazine-H), 8.52 (d, 1H, J = 1.5 Hz, pyrazine-H), 7.72 (d, 1H, J = 8.0 Hz, NH), 4.19-4.03 (m, 3H, CH+CH_2), 2.95 (t, 2H, J = 13.5 Hz, CH_2), 2.00 (d, 2H, J = 12.5 Hz, CH_2), 1.54-1.43 (m, 11H, CH_2+CH_3); ESI-MS: m/z = 307.2 $[M+H]^+$.

4.1.2 General procedure for the synthesis of protected piperidine fragments (**8a-g**)

To a mixture of *N*-Boc-4-piperidinecarboxylic acid (3.65g, 16.0mmol), pyridine (3.4mL, 40.0mmol) and CH_2Cl_2 (30.0mL), $SOCl_2$ (1.5mL, 19.0mmol) was added under N_2 at room temperature. The mixture was stirred for half an hour, and a solution of corresponding amine (**6a-g**, 18.0mmol), Et_3N (8.0mL, 56.0mmol) and a catalytic amount of DMAP in CH_2Cl_2 (30.0mL) was added dropwise. The suspension was stirred overnight. The organic phase was washed with 1N HCl (2×30mL) and aqueous $NaHCO_3$ (2×30mL), dried over Na_2SO_4 (For compounds **8e** and **8f**, the solvent was evaporated and washed with aqueous $NaHCO_3$). The solvent was evaporated under reduced pressure and the crude product was purified by flash column chromatography (ethyl acetate: petroleum ether = 1:2~3:1) to give compounds **8a-g**.

4.1.2.1 **tert-Butyl 4-(Pyrazin-2-ylcarbamoyl) piperidine-1-carboxylate (8a)**

White solid; Yield: 78%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 9.55 (s, 1H, pyrazine-H), 8.35 (d, 1H, J = 2.0 Hz, pyrazine-H), 8.23 (s, 1H, pyrazine-H), 7.97 (s, 1H, NH), 4.24-4.15 (m, 2H, CH_2), 2.86-2.75 (m, 2H, CH_2), 2.51-4.46 (m, 1H, CH), 1.93 (d, 2H, J = 12.5 Hz, CH_2), 1.80-1.72 (m, 2H, CH_2), 1.47 (s, 9H,

CH_3); ESI-MS: m/z = 307.2 $[M+H]^+$.

4.1.2.2 **tert-Butyl 4-(Phenylcarbamoyl) piperidine-1-carboxylate (8b)**

White solid; Yield: 86%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.53 (d, 2H, *J* = 5.0 Hz, Ar-H), 7.39-7.30 (m, 3H, Ar-H+NH), 7.15-7.09 (m, 1H, Ar-H), 4.21 (d, 2H, *J* = 10.0 Hz, CH_2), 2.85-2.74 (m, 2H, CH_2), 2.44-2.38 (m, 1H, CH), 1.94-1.87 (m, 2H, CH_2), 1.77-1.69 (m, 2H, CH_2), 1.48 (s, 9H, CH_3); ESI-MS: m/z = 305.2 $[M+H]^+$.

4.1.2.3 **tert-Butyl 4-(4-Fluorophenylcarbamoyl) piperidine-1-carboxylate (8c)**

White solid; Yield: 75%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.50-7.42 (m, 2H, Ar-H), 7.20 (s, 1H, NH), 7.01 (t, 2H, *J* = 8.0 Hz, Ar-H), 4.18 (d, 2H, *J* = 12.0 Hz, CH_2), 2.80 (t, 2H, *J* = 12.5 Hz, CH_2), 2.39-2.32 (m, 1H, CH), 1.90 (d, 2H, *J* = 12.5 Hz, CH_2), 1.79-1.70 (m, 2H, CH_2), 1.47 (s, 9H, CH_3); ESI-MS: m/z = 323.3 $[M+H]^+$.

4.1.2.4 **tert-Butyl 4-(4-Methoxyphenylcarbamoyl) piperidine-1-carboxylate (8d)**

White solid; Yield: 85%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.39 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.38 (s, 1H, NH), 6.83 (d, 2H, *J* = 7.5 Hz, Ar-H), 4.16 (d, 2H, *J* = 13.0 Hz, CH_2), 3.77 (s, 3H, CH_3), 2.81-2.69 (m, 2H, CH_2), 2.38-2.32 (m, 1H, CH), 1.86 (d, 2H, *J* = 12.0 Hz, CH_2), 1.76-1.71 (m, 2H, CH_2), 1.45 (s, 9H, CH_3); ESI-MS: m/z = 335.2 $[M+H]^+$.

4.1.2.5 **tert-Butyl 4-(Pyridin-2-ylcarbamoyl) piperidine-1-carboxylate (8e)**

White solid; Yield: 65%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.27-8.22 (m, 3H, pyridine-H+NH), 7.75-7.71 (m, 1H, pyridine-H), 7.06 (dd, 1H, *J* = 6.5, 5.0 Hz, pyridine-H), 4.18 (brs, 2H, CH_2), 2.83-2.70 (m, 2H, CH_2), 2.45-2.38 (m, 1H, CH), 1.91 (d, 2H, *J* = 12.5 Hz, CH_2), 1.78-1.69 (m, 2H, CH_2), 1.46 (s, 9H, CH_3); ESI-MS: m/z = 306.2 $[M+H]^+$.

4.1.2.6 **tert-Butyl 4-(Pyridin-3-ylcarbamoyl) piperidine-1-carboxylate (8f)**

White solid; Yield: 63%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.61 (s, 1H, pyridine-H), 8.34 (d, 1H, *J* = 4.0 Hz, pyridine-H), 8.28 (d, 1H, *J* = 8.5 Hz, pyridine-H), 7.81 (s, 1H, NH), 7.32 (dd, 1H, *J* = 8.5, 5.0 Hz, pyridine-H), 4.19 (d, 2H, *J* = 13.0 Hz, CH_2), 2.90-2.71 (m, 2H, CH_2), 2.49-2.43 (m, 1H, CH), 1.91 (d, 2H, *J* = 12.0 Hz, CH_2), 1.77-1.73 (m, 2H, CH_2), 1.46 (s, 9H, CH_3); ESI-MS: m/z = 306.2 $[M+H]^+$.

4.1.2.7 **tert-Butyl 4-(Thiazol-2-ylcarbamoyl) piperidine-1-carboxylate (8g)**

White solid; Yield: 87%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 12.10 (s, 1H, NH), 7.42 (d, 1H, *J* = 4.0 Hz, thiazole-H), 7.05 (d, 1H, *J* = 3.5 Hz, thiazole-H), 4.29-4.11 (m, 2H, CH_2), 2.93-2.80 (m, 2H, CH_2), 2.67-2.62 (m, 1H, CH), 1.91 (d, 2H, *J* = 11.5 Hz, CH_2), 1.87-1.80 (m, 2H, CH_2), 1.48 (s, 9H, CH_3);

ESI-MS: m/z = 312.1 $[M+H]^+$.

4.1.3 General procedure for the synthesis of piperidine contained fragments (**9**, **10a-g**)

To a solution of protected piperidine fragments **7** or **8a-g** (5.0mmol) in CH_2Cl_2 (20.0mL) was added trifluoroacetic acid (TFA, 5.0mL) at 0 ^oC. The reaction mixture was then warmed to room temperature and stirred for 1h. The volatiles were evaporated under reduced pressure and ether (30.0mL) was added. White solid was precipitated, filtrated and dried, which was put into next step without further purification.

4.1.4 **tert-Butyl-hPhe-2-chlorobenzylamide (12)**

To a suspension of Boc-L-hPhe (1.40g, 5.0mmol) in DCM (20.0mL), HOBt (0.74g, 5.5mmol) and EDCI (1.44g, 7.5mmol) were added at 0^oC. The reaction mixture was stirred for 30min. Then (2-chlorophenyl)methanamine (**11**, 0.71g, 5.0mmol) and diisopropylethylamine (1.9mL, 10.0mmol) were added. After stirring at room temperature for 2h, the resulting mixture was washed with aqueous $NaHCO_3$ solution (1×30mL), brine (1×30mL) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and the crude product was purified by flash chromatography (ethyl acetate: petroleum ether = 1:4).

White solid; Yield: 89%; ^1H NMR (500MHz, $CDCl_3$): δ = 7.37-7.34 (m, 2H, Ar-H), 7.28-7.25 (m, 2H, Ar-H), 7.24-7.17 (m, 3H, Ar-H), 7.13 (d, 2H, J = 7.0 Hz, Ar-H), 6.52 (t, 1H, J = 5.0 Hz, NH), 5.02 (d, 1H, J = 6.5 Hz, NH), 4.53 (d, 2H, J = 6.0 Hz, CH_2), 4.10-4.08 (m, 1H, CH), 2.70-2.60 (m, 2H, CH_2), 2.21-2.14 (m, 1H, CH_2), 1.96-1.88 (m, 1H, CH_2), 1.43 (s, 9H, CH_3); ESI-MS: m/z = 403.2 $[M+H]^+$.

4.1.5 **hPhe-2-chlorobenzylamide TFA salt (13)**

To a solution of compound **12** (5.0mmol) in CH_2Cl_2 (20.0mL) was added TFA (5.0mL) at 0 ^oC. The reaction mixture was then warmed to room temperature and stirred for 1h. The volatiles were evaporated under reduced pressure and ether (30.0mL) was added. White solid was precipitated, filtrated and dried, which was put into next step without further purification.

4.1.6 General procedure for the synthesis of protected dipeptide fragments (**14a-d**)

To a solution of corresponding amino-protected amino acid (5.0mmol) in DCM (20.0mL), HOBt (0.74g, 5.5mmol) and EDCI (1.44g, 7.5mmol) were added at 0^oC. The reaction mixture was stirred for 30min. Then compound **13** (1.87g, 4.5mmol) and diisopropylethylamine (1.9mL, 10.0mmol) were added. After stirring at room temperature for 3h, the resulting mixture was washed with aqueous $NaHCO_3$ solution (1×30mL), brine (1×30mL) and dried over Na_2SO_4. The solvent was evaporated

under reduced pressure and the crude product was purified by flash chromatography (ethyl acetate: petroleum ether = 1:3~2:1).

4.1.6.1 **tert-Butyl-Ala-hPhe-2-chlorobenzylamide (14a)**

White solid; Yield: 92%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.36-7.27 (m, 4H, Ar-H), 7.24-7.13 (m, 5H, Ar-H), 6.75 (brs, 1H, NH), 6.54 (brs, 1H, NH), 4.75 (brs, 1H, NH), 4.53-4.45 (m, 3H, CH_2+CH), 4.07 (brs, 1H, CH), 2.67 (m, 2H, CH_2), 2.26-2.25 (m, 1H, CH_2), 2.03 (m, 1H, CH_2), 1.40 (s, 9H, CH_3), 1.30 (d, 3H, J = 6.5 Hz, CH_3); ESI-MS: m/z = 474.3 $[M+H]^+$.

4.1.6.2 **tert-Butyl-Leu-hPhe-2-chlorobenzylamide (14b)**

White solid; Yield: 71%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.35-7.27 (m, 2H, Ar-H), 7.24-7.12 (m, 7H, Ar-H), 6.81 (brs, 1H, NH), 6.57 (brs, 1H, NH), 4.72 (brs, 1H, NH), 4.55-4.45 (m, 3H, CH_2+CH), 4.03 (brs, 1H, CH), 2.65 (m, 2H, CH_2), 2.24-2.22 (m, 1H, CH_2), 2.02 (m, 1H, CH_2), 1.60 (m, 2H, CH_2), 1.43-1.41 (m, 1H, CH), 1.39 (s, 9H, CH_3), 0.91 (t, 6H, J = 8.0 Hz, CH_3); ESI-MS: m/z = 516.3 $[M+H]^+$.

4.1.6.3 **tert-Butyl- Phe-hPhe-2-chlorobenzylamide (14c)**

White solid; Yield: 88%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.35-7.33 (m, 1H, Ar-H), 7.30-7.27 (m, 3H, Ar-H), 7.24-7.13 (m, 8H, Ar-H), 7.10-7.08 (d, 2H, J = 7.0 Hz, Ar-H), 6.61 (brs, 1H, NH), 6.36 (brs, 1H, NH), 4.80 (brs, 1H, NH), 4.54-4.40 (m, 3H, CH+CH_2), 4.30-4.27 (m, 1H, CH), 3.04 (dd, 1H, J = 13.5, 6.0 Hz, CH_2), 3.02 (dd, 1H, J = 13.5, 6.0 Hz, CH_2), 2.63-2.56 (m, 2H, CH_2), 2.20-2.11 (m, 1H, CH_2), 1.97-1.92 (m, 1H, CH_2), 1.36 (s, 9H, CH_3); ESI-MS: m/z = 550.3 $[M+H]^+$.

4.1.6.4 **tert-Butyl-hPhe-hPhe-2-chlorobenzylamide (14d)**

White solid; Yield: 87%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.33-7.26 (m, 4H, Ar-H), 7.25-7.11 (m, 10H, Ar-H), 6.72 (brs, 1H, NH), 6.54 (brs, 1H, NH), 4.85 (brs, 1H, NH), 4.55-4.41 (m, 3H, CH+CH_2), 4.01 (brs, 1H, CH), 2.67-2.61 (m, 4H, CH_2), 2.24-2.18 (m, 1H, CH_2), 2.11-1.96 (m, 2H, CH_2), 1.87-1.79 (m, 1H, CH_2), 1.41 (s, 9H, CH_3); ESI-MS: m/z = 564.3 $[M+H]^+$.

4.1.7 General procedure for the synthesis of deprotected dipeptide fragments (**15a-d**)

To a solution of corresponding dipeptide fragment **14a-d** (3.0mmol) in CH_2Cl_2 (10.0mL) was added TFA (3.0mL) at 0 ^{o}C. The reaction mixture was then warmed to room temperature and stirred for 1h. The volatiles were evaporated under reduced pressure and ether (20.0mL) was added. White solid was precipitated, filtrated and dried, which was put into next step without further purification.

4.1.8 General procedure for the synthesis of target compounds **16-29**

Corresponding dipeptide fragment **15a-d** (3.0mmol) was dissolved in CH_2Cl_2 (5.0mL) and saturated aqueous $NaHCO_3$ (5.0mL) and was cooled to 0 ^oC. Triphosgene (0.30g, 1.0mmol) was added in one portion and the mixture was stirred at the same temperature for 15min. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2×10mL). Then the organic phase was combined, dried over Na_2SO_4, evaporated in vacuo and used directly. To a solution of corresponding piperidine contained TFA salt (**9**, **10a-g**, 2.0mmol) was added diisopropylethylamine (0.8mL, 4.0mmol). Then the former obtained isocyanate was dissolved in CH_2Cl_2 (3.0mL) and added into the reaction mixture. After 3h's stirring, the mixture was washed with saturated aqueous $NaHCO_3$, NH_4Cl and brine. The organic phase was dried over Na_2SO_4, evaporated to obtain the crude product. Purification was accomplished by column chromatography (dichloromethane: methanol=50:1~20:1). Analysis of sample purity was performed on an Agilent 1200 HPLC system using an Agilent ZORBAX SB-Aq, 5 μM C18 column (250 mm × 4.6 mm). HPLC condition: linear density gradient elution with eluents MeOH/H_2O = 75/25-95/5 (10 min), flow rate: 1.0 mL/min.

4.1.8.1 **4-(Pyrazin-2-ylcarbamoyl) piperidine-1-carbonyl-Ala-hPhe-2-chlorobenzylamide (16)**

White solid; Yield: 36%; mp: 133-135 ^oC; Purity: 98.7%; ^1H NMR (500MHz, d_6-DMSO): δ = 10.76 (s, 1H, NH), 9.34 (d, 1H, *J* = 1.5 Hz, pyrazine-H), 8.41-8.38 (m, 2H, NH+pyrazine-H), 8.35 (d, 1H, *J* = 2.5 Hz, pyrazine-H), 8.00 (d, 1H, *J* = 8.0 Hz, NH), 7.44-7.42 (m, 1H, Ar-H), 7.33-7.27 (m, 5H, Ar-H), 7.19-7.16 (m, 3H, Ar-H), 6.65 (d, 1H, *J* = 6.5 Hz, NH), 4.34 (d, 2H, *J* = 6.0 Hz, CH_2), 4.27-4.23 (m, 1H, CH), 4.15 (m, 1H, CH), 4.04 (d, 2H, *J* = 12.5 Hz, CH_2), 2.75-2.53 (m, 5H, CH+CH_2), 2.08-1.99 (m, 1H, CH), 1.95-1.87 (m, 1H, CH), 1.77 (d, 2H, *J* = 5.0 Hz, CH_2), 1.56-1.45 (m, 2H, CH_2), 1.28 (d, 3H, *J* = 7.0 Hz, CH_3); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.71, 174.27, 172.13, 157.71, 149.36, 143.03, 141.86, 140.06, 136.75, 136.66, 132.43, 129.50, 129.16, 129.03, 128.81, 128.78, 127.54, 126.28, 52.74, 50.85, 43.52, 43.48, 42.54, 33.95, 31.85, 28.44, 28.39, 18.14; ESI-MS: m/z = 606.0 $[M+H]^+$.

4.1.8.2 **4-(Pyrazine-2-carboxamido) piperidine-1-carbonyl-Ala-hPhe-2-chlorobenzylamide (17)**

White solid; Yield: 29%; mp: 154-156 ^oC; Purity: 99.1%; ^1H NMR (500MHz, d_6-DMSO): δ = 9.18 (d, 1H, *J* = 1.5 Hz, pyrazine-H), 8.86 (d, 1H, *J* = 2.5 Hz, pyrazine-H), 8.75 (d, 1H, *J* = 8.5 Hz, NH), 8.70 (dd, 1H, *J* = 2.5, 1.0 Hz , pyrazine-H), 8.43 (t, 1H, *J* = 12.0 Hz, NH), 7.96 (d, 1H, *J* = 7.5 Hz, NH), 7.44-7.42 (m, 1H, Ar-H), 7.33-7.27 (m, 5H, Ar-H), 7.19-7.16 (m, 3H, Ar-H), 6.69 (d, 1H, *J* = 6.5 Hz, NH), 4.36 (d, 2H, *J* = 6.0 Hz, CH_2), 4.29-4.25 (m, 1H, CH), 4.16-4.10 (m, 1H, CH_2), 4.05-3.97 (m, 3H, *J* = 12.0 Hz, CH_2), 2.83-2.74 (m, 2H, CH_2), 2.64-2.54 (m, 2H, CH_2), 2.08-2.00 (m, 1H, CH),

1.95-1.87 (m, 1H, CH), 1.76-1.70 (m, 2H, CH_2), 1.63-1.46 (m, 2H, CH), 1.29 (d, 3H, J = 7.0 Hz, CH_3); ^{13}C NMR (100MHz, d_6-DMSO):δ = 174.23, 172.46, 172.11, 162.62, 157.64, 147.87, 145.39, 144.06, 143.68, 141.88, 136.62, 132.46, 129.52, 129.22, 129.06, 128.79, 127.56, 126.29, 52.70, 50.98, 47.12, 43.30, 43.20, 34.11, 31.84, 31.48, 18.18; ESI-MS: m/z = 606.0 $[M+H]^+$.

4.1.8.3 **4-(Pyrazin-2-ylcarbamoyl) piperidine-1-carbonyl-Leu-hPhe-2-chlorobenzylamide (18)**

White solid; Yield: 26%; mp: 116-118 ^{o}C; Purity: 98.5%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.77 (s, 1H, NH), 9.34 (d, 1H, J = 1.0 Hz, pyrazine-H), 8.40-8.35 (m, 3H, pyrazine-H+NH), 8.01 (d, 1H, J = 7.5 Hz, NH), 7.44-7.42 (m, 1H, Ar-H), 7.33-7.27 (m, 5H, Ar-H), 7.19-7.16 (m, 3H, Ar-H), 6.58 (d, 1H, J = 7.5 Hz, NH), 4.34 (d, 2H, J = 6.0 Hz, CH_2), 4.27-4.23 (m, 1H, CH), 4.18-4.13 (m, 1H, CH), 4.06-4.04 (m, 2H, CH_2), 2.75-2.58 (m, 5H, CH+CH_2), 2.07-1.85 (m, 2H, CH_2), 1.76 (d, 2H, J = 12.0 Hz, CH_2), 1.71-1.64 (m, 1H, CH), 1.64-1.44 (m, 4H, CH_2), 0.89 (dd, 6H, J = 19.0, 6.5 Hz, CH_3); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.71, 174.05, 172.06, 157.87, 149.36 143.03, 141.84, 140.06, 136.76, 136.63, 132.47, 129.52, 129.22, 129.07, 128.78, 127.52, 126.29, 53.63, 52.74, 43.67, 43.61, 42.57, 33.96, 31.79, 28.44, 28.39, 24.81, 23.53, 22.14; ESI-MS: m/z = 648.1 $[M+H]^+$.

4.1.8.4 **4-(Pyrazine-2-carboxamido) piperidine-1-carbonyl-Leu-hPhe-2-chlorobenzylamide (19)**

White solid; Yield: 38%; mp: 141-143 ^{o}C; Purity: 98.8%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 9.18 (d, 1H, J = 1.0 Hz, pyrazine-H), 8.86 (d, 1H, J = 1.0 Hz, pyrazine-H), 8.79 (d, 1H, J = 8.0 Hz, NH), 8.69 (s, 1H, pyrazine-H), 8.43 (t, 1H, J = 5.5 Hz, NH), 8.00 (d, 1H, J = 7.5 Hz, NH), 7.43-7.37 (m, 1H, Ar-H), 7.31-7.27 (m, 5H, Ar-H), 7.19-7.16 (m, 3H, Ar-H), 6.63 (d, 1H, J = 7.0 Hz, NH), 4.35 (d, 2H, J = 5.5 Hz, CH_2), 4.28-4.21 (m, 1H, CH), 4.15-4.11 (m, 1H, CH_2), 4.05-3.94 (m, 3H, CH_2), 2.82-2.73 (m, 2H, CH_2), 2.64-2.53 (m, 2H, CH_2), 2.06-1.97 (m, 1H, CH), 1.95-1.87 (m, 1H, CH), 1.73-1.68 (m, 3H, CH+CH_2), 1.63-1.58 (m, 2H, CH_2), 1.50-1.44 (m, 2H, CH_2), 0.80 (dd, 6H, J = 18.5, 6.0 Hz, CH_3); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.03, 172.05, 162.62, 157.80, 147.88, 145.41, 144.06, 143.67, 141.86, 136.59, 132.49, 129.54, 129.27, 129.10, 128.80, 128.76, 127.55, 126.31, 53.79, 52.70, 47.16, 43.43, 43.29, 34.12, 31.78, 31.50, 24.80, 23.53, 22.15; m/z = 648.1 $[M+H]^+$.

4.1.8.5 **4-(Pyrazin-2-ylcarbamoyl) piperidine-1-carbonyl-Phe-hPhe-2-chlorobenzylamide (20)**

White solid; Yield: 42%; mp: 110-112 ^{o}C; Purity: 99.3%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.74 (s, 1H, NH), 9.33 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.40-8.39 (m, 1H, pyrazine-H), 8.36-8.33 (m, 2H, NH+pyrazine-H), 8.19 (d, 1H, J = 3.0 Hz, NH), 7.45-7.43 (m, 1H, Ar-H), 7.33-7.25 (m, 9H, Ar-H), 7.20-7.17 (m, 4H, Ar-H), 6.71 (d, 1H, J = 8.0 Hz, NH), 4.39-4.34 (m, 3H, CH_2+CH), 4.29-4.23 (m, 1H,

CH), 4.01-3.96 (m, 2H, CH_2), 3.06-3.02 (dd, 1H, J = 14.0, 4.5 Hz, CH_2), 2.94-2.89 (dd, 1H, J = 13.5, 9.5 Hz, CH_2), 2.70-2.51 (m, 5H, CH+CH_2), 2.07-1.99 (m, 1H, CH_2), 1.95-1.85 (m, 1H, CH_2), 1.74-1.71 (m, 2H, CH_2), 1.48-1.34 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.66, 173.22, 172.03, 157.64, 149.35, 143.03, 141.84, 140.05, 139.19, 136.75, 136.63, 129.72, 129.53, 129.15, 129.06, 128.81, 128.78, 128.45, 127.55, 126.55, 126.29, 56.56, 52.91, 43.59, 43.50, 42.51, 37.25, 34.16, 31.76, 28.33; ESI-MS: m/z = 682.1 $[M+H]^+$.

4.1.8.6 **4-(Pyrazine-2-carboxamido) piperidine-1-carbonyl-Phe-hPhe-2-chlorobenzylamide (21)**

White solid; Yield: 30%; mp: 105-107 ^{o}C; Purity: 97.9%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 9.18 (d, 1H, J = 1.0 Hz, pyrazine-H), 8.87 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.70 (m, 2H, J = 2.5 Hz, NH+pyrazine-H), 8.36 (t, 1H, J = 6.0 Hz, NH), 8.18(d, 1H, J = 7.5 Hz, NH), 7.45-7.43 (m, 1H, Ar-H), 7.33-7.26 (m, 8H, Ar-H), 7.20-7.17 (m, 3H, Ar-H), 6.75 (d, 1H, J = 8.0 Hz, NH), 4.37-4.33 (m, 2H, CH_2), 4.30-4.26 (m, 1H, CH), 3.98-3.92 (m, 2H, CH_2), 3.04 (dd, 1H, J = 14.0, 4.5 Hz, CH_2), 2.92 (dd, 1H, J = 13.5, 10.0 Hz, CH_2), 2.77-2.68 (m, 2H, CH_2), 2.65-2.55 (m, 2H, CH_2), 2.07-1.99 (m, 1H, CH), 1.96-1.89 (m, 1H, CH), 1.69 (brs, 2H, CH_2), 1.56-1.48 (m, 1H, CH_2), 1.41-1.24 (m, 3H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO):δ= 173.20, 172.02, 162.61, 157.61, 147.89, 145.38, 144.05, 143.69, 141.85, 139.19, 136.60, 132.46, 129.72, 129.54, 129.19, 129.08, 128.80, 128.48, 127.56, 126.56, 126.30, 56.68, 52.91, 47.10, 43.28, 43.18, 37.30, 34.24, 31.76, 31.44; ESI-MS: m/z = 682.1 $[M+H]^+$.

4.1.8.7 **4-(Pyrazin-2-ylcarbamoyl) piperidine-1-carbonyl-hPhe-hPhe-2-chlorobenzylamide (22)**

White solid; Yield: 43%; mp: 178-180 ^{o}C; Purity: 98.4%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.77 (s, 1H, NH), 9.34 (s, 1H, pyrazine-H), 8.43-8.39 (m, 2H, pyrazine-H+NH), 8.35 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.07 (d, 1H, J = 7.5 Hz, NH), 7.41 (d, 1H, J = 7.0 Hz, Ar-H), 7.33-7.16 (m, 13H, Ar-H), 6.68 (d, 1H, J = 7.5 Hz, NH), 4.36-4.27 (m, 3H, CH+CH_2), 4.16-4.03 (m, 3H, CH+CH_2), 2.78-2.55 (m, 7H, CH+CH_2), 2.05-1.94 (m, 4H, CH_2), 1.80 (d, 2H, J = 11.5 Hz, CH_2), 1.59-1.46 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.72, 173.55, 172.45, 172.10, 157.89, 149.37, 143.02, 142.21, 141.83, 140.06, 136.77, 136.63, 132.44, 129.49, 129.17, 129.00, 128.79, 128.74, 127.50, 126.29, 126.22, 55.03, 52.81, 43.68, 43.63, 33.98, 33.83, 32.40, 31.85, 28.45, 21.51; ESI-MS: m/z = 696.1 $[M+H]^+$.

4.1.8.8 **4-(Pyrazine-2-carboxamido) piperidine-1-carbonyl-hPhe-hPhe-2-chlorobenzylamide (23)**

White solid; Yield: 39%; mp: 104-105 ^{o}C; Purity: 99.1%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 9.19 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.86 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.77 (d, 1H, J = 8.5 Hz, NH), 8.69

(d, 1H, *J* = 2.5 Hz, pyrazine-H), 8.44 (t, 1H, *J* = 6.0 Hz, NH), 8.03 (d, 1H, *J* = 7.0 Hz, NH), 7.41-7.39 (m, 1H, Ar-H), 7.32-7.24 (m, 7H, Ar-H), 7.22-7.17 (m, 6H, Ar-H), 6.71 (d, 1H, *J* = 7.0 Hz, NH), 4.35-4.28 (m, 3H, CH+CH_2), 4.15-4.09 (m, 1H, CH), 4.07-4.02 (m, 3H, CH_2+CH), 2.86-2.54 (m, 6H, CH_2), 2.05-1.90 (m, 4H, CH_2), 1.75 (d, 2H, *J* = 11.5 Hz, CH_2), 1.63-1.51 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.53, 172.09, 162.63, 157.81, 147.87, 145.41, 144.07, 143.67, 142.21, 141.84, 136.59, 132.47, 129.51, 129.22, 129.04, 128.80, 127.52, 126.31, 126.23, 55.18, 52.78, 47.18, 43.44, 43.33, 34.14, 33.89, 32.44, 31.84, 31.53, 21.51; ESI-MS: m/z = 696.1 $[M+H]^+$.

4.1.8.9 **4-(Phenylcarbamoyl) piperidine-1-carbonyl-hPhe-hPhe-2-chlorobenzylamide (24)**

White solid; Yield: 58%; mp: 154-156 ^{o}C; Purity: 98.6%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 9.92 (s, 1H, NH), 8.44 (t, 1H, *J* = 6.0 Hz, NH), 8.10 (d, 1H, *J* = 7.5 Hz, NH), 7.61 (d, 2H, *J* = 8.0 Hz, Ar-H), 7.42-7.39 (m, 1H, Ar-H), 7.32-7.18 (m, 15H, Ar-H), 7.03 (t, 1H, *J* = 7.5 Hz, Ar-H), 6.70 (d, 1H, *J* = 7.5 Hz, NH), 4.32 (d, 2H, *J* = 6.0 Hz, CH_2), 4.29-4.23 (m, 1H, CH), 4.16-4.04 (m, 3H, CH+CH_2), 2.78-2.57 (m, 7H, CH+CH_2), 2.03-1.90 (m, 4H, CH_2), 1.76 (d, 2H, *J* = 11.0 Hz, CH_2), 1.59-1.45 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.59, 173.56, 172.10, 157.90, 142.22, 141.82, 139.79, 136.62, 132.44, 129.50, 129.17, 129.10, 129.01, 128.79, 128.75, 127.51, 126.30, 126.23, 123.50, 119.61, 55.03, 52.81, 43.73, 43.69, 43.27, 33.97, 33.85, 32.40, 31.85, 28.67; ESI-MS: m/z = 684.1 $[M+H]^+$.

4.1.8.10 **4-(4-Fluorophenylcarbamoyl) piperidine-1-carbonyl-hPhe-hPhe-2-chlorobenzylamide (25)**

White solid; Yield: 49%; mp: 148-150 ^{o}C; Purity: 98.6%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.74 (m, 2H, NH+NH), 7.29-7.27 (m, 2H, Ar-H), 7.25-7.13 (m, 10H, Ar-H), 7.10-7.02 (m, 6H, Ar-H), 6.28 (d, 1H, *J* = 8.0 Hz, NH), 4.98 (d, 1H, *J* = 5.0 Hz, NH), 4.52-4.47 (m, 2H, CH_2+CH), 4.42 (dd, 1H, *J* = 15.0, 6.0 Hz, CH_2), 4.32-4.29 (m, 1H, CH), 4.12-4.06 (m, 1H, CH), 3.80 (d, 1H, *J* = 13.5 Hz, CH_2), 3.47 (d, 1H, *J* = 13.5 Hz, CH_2), 2.79-2.60 (m, 6H, CH_2), 2.24-2.11 (m, 2H, CH_2), 2.04-1.98 (m, 2H, CH_2), 1.91-1.84 (m, 2H, CH_2), 1.45-1.37 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.54, 172.10, 165.49, 165.01, 163.03, 157.84, 142.22, 141.83, 136.59, 132.45, 131.59, 131.56, 130.42, 130.33, 129.56, 129.51, 129.18, 129.09, 129.04, 128.78, 127.52, 126.32, 126.24, 115.63, 115.42, 55.09, 52.82, 47.35, 43.45, 43.29, 34.04, 33.95, 32.43, 31.18, 31.79; ESI-MS: m/z = 712.1 $[M+H]^+$.

4.1.8.11 **4-(4-Methoxyphenylcarbamoyl) piperidine-1-carbonyl-hPhe-hPhe-2-chlorobenzylamide (26)**

White solid; Yield: 45%; mp: 168-169 ^{o}C; Purity: 98.2%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 7.42-7.28 (m, 8H, NH+Ar-H), 7.26-7.12 (m, 10H, NH+Ar-H), 7.01-6.97 (brs, 1H, NH), 6.84 (d, 2H, J = 8.5 Hz, Ar-H), 4.51-4.47 (m, 3H, CH+CH_2), 4.25 (brs, 1H, CH), 3.78 (s, 3H, CH_3), 3.65-3.58 (m, 2H, CH_2), 2.75-2.64 (m, 6H, CH_2), 2.37-2.34 (m, 1H, CH), 2.28-2.21 (m, 1H, CH_2), 2.19-2.12 (m, 1H, CH_2), 2.07-1.96 (m, 2H, CH_2), 1.90-1.80 (m, 2H, CH_2), 1.73-1.65 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.57, 173.07, 172.11, 157.90, 155.55, 142.22, 141.83, 136.63, 132.96, 132.45, 129.50, 129.17, 129.01, 128.79, 128.75, 127.50, 126.30, 126.23, 121.17, 114.24, 55.62, 55.04, 52.82, 43.76, 43.70, 43.16, 33.97, 33.85, 32.41, 31.85, 28.71; ESI-MS: m/z = 724.1 $[M+H]^+$.

4.1.8.12 **4-(Pyridin-2-ylcarbamoyl) piperidine-1-carbonyl-hPhe-hPhe-2-chlorobenzylamide (27)**

White solid; Yield: 39%; mp: 166-167 ^{o}C; Purity: 98.1%; ^{1}H NMR (500MHz, $CDCl_3$): δ = 8.73 (brs, 1H, NH), 8.26-8.23 (m, 2H, pyridine-H), 7.78 (t, 1H, J = 8.5 Hz, NH), 7.34-7.28 (m, 4H, Ar-H+pyridine-H), 7.25-7.03 (m, 12H, Ar-H+pyridine-H), 6.76 (d, 1H, J = 8.0 Hz, NH), 4.80 (d, 1H, J = 6.0 Hz, NH), 4.55-4.49 (m, 3H, CH+CH_2), 4.23 (m, 1H, CH), 3.72 (d, 1H, J = 13.0 Hz, CH_2), 3.60 (d, 1H, J = 13.0 Hz, CH_2), 2.80-2.60 (m, 6H, CH_2), 2.46-2.39 (m, 1H, CH), 2.33-2.22 (m, 1H, CH_2), 2.17-2.12 (m, 1H, CH_2), 2.01-1.95 (m, 2H, CH_2), 1.87-1.78 (m, 2H, CH_2), 1.72-1.60 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.53, 173.57, 172.11, 157.89, 152.60, 148.33, 142.21, 141.82, 138.55, 136.62, 132.44, 129.49, 129.17, 129.01, 128.79, 128.75, 127.50, 126.30, 126.23, 119.73, 114.00, 55.03, 52.81, 43.73, 43.67, 42.76, 33.96, 33.83, 32.40, 31.85, 28.55, 28.52; ESI-MS: m/z = 695.3 $[M+H]^+$.

4.1.8.13 **4-(Pyridin-3-ylcarbamoyl) piperidine-1-carbonyl-hPhe-hPhe-2-chlorobenzylamide (28)**

White solid; Yield: 52%; mp: 141-143 ^{o}C; Purity: 98.5%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 10.17 (s, 1H, NH), 8.77 (d, 1H, J = 2.5 Hz, pyridine-H), 8.44 (t, 1H, J = 6.0 Hz, NH), 8.26 (dd, 1H, J = 4.5, 1.5 Hz, pyridine-H), 8.10-8.06 (m, 2H, NH+pyridine-H), 7.40 (dd, 1H, J = 9.5, 2.0 Hz, pyridine-H), 7.37-7.17 (m, 14H, Ar-H, pyridine-H), 6.69 (d, 1H, J = 6.5 Hz, NH), 4.33 (d, 2H, J = 6.0 Hz, CH_2), 4.30-4.26 (m, 1H, CH), 4.17-4.04 (m, 3H, CH+CH_2), 2.80-2.68 (m, 3H, CH+CH_2), 2.66-2.53 (m, 4H, CH_2), 2.06-1.88 (m, 4H, CH_2), 1.78 (d, 2H, J = 11.0 Hz, CH_2), 1.57-1.49 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 174.17, 173.55, 172.10, 157.89, 142.21, 141.82, 141.19, 136.62, 136.40, 132.43, 129.50, 129.16, 129.11, 129.02, 128.80, 128.76, 127.51, 126.61, 126.31, 126.24, 124.08, 55.01, 52.80, 43.67, 43.62, 43.16, 33.97, 33.85, 32.40, 31.84, 28.55; ESI-MS: m/z = 695.3 $[M+H]^+$.

4.1.8.14 **4-(Thiazol-2-ylcarbamoyl) piperidine-1-carbonyl-hPhe-hPhe-2-chlorobenzylamide (29)**

White solid; Yield: 32%; mp: 144-145 ^{o}C; Purity: 98.9%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 12.1 (s, 1H, NH), 8.43 (t, 1H, J = 6.0 Hz, NH), 8.09 (d, 1H, J = 8.0 Hz, NH), 7.47 (d, 1H, J = 3.5 Hz, thiazole-H), 7.42-7.40 (m, 1H, Ar-H), 7.32-7.17 (m, 14H, thiazole-H+Ar-H), 6.69 (d, 1H, J = 7.5 Hz, NH), 4.33 (d, 2H, J = 6.0 Hz, CH_2), 4.30-4.24 (m, 1H, CH), 4.17-4.02 (m, 3H, CH+CH_2), 2.79-2.54 (m, 7H, CH+CH_2), 1.99-1.92 (m, 4H, CH_2), 1.80 (d, 2H, J = 10.5 Hz, CH_2), 1.59-1.46 (m, 2H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 173.54, 173.47, 172.10, 158.47, 157.88, 142.21, 141.82, 138.02, 136.62, 132.44, 129.49, 129.18, 129.01, 128.79, 128.75,127.50, 126.30, 126.23, 113.83, 55.03, 52.81, 43.61, 43.58, 41.84, 33.96, 33.84, 32.40, 31.84, 28.28; ESI-MS: m/z = 701.2 $[M+H]^+$.

4.1.8.15 **3-Pyridinyl-carbonyl-hPhe-hPhe-2-chlorobenzylamide**

White solid; Yield: 76%; mp: 138-140 ^{o}C; Purity: 98.5%; ^{1}H NMR (500MHz, d_6-DMSO): δ = 9.08 (d, 1H, J = 2.0 Hz, pyridine-H), 8.87 (d, 1H, J = 9.0 Hz, NH), 8.74 (dd, 1H, J = 6.0, 1.5 Hz, pyridine-H), 8.40 (t, 1H, J = 7.5 Hz, NH), 8.30-8.23 (m, 2H, NH+pyridine-H), 7.55-7.52 (m, 1H, Ar-H), 7.41 (dd, 1H, J = 9.5, 2.5 Hz, pyridine-H), 7.33-7.17 (m, 13H, Ar-H), 4.58-4.52 (m, 1H, CH), 4.37-4.32 (m, 3H, CH+CH_2), 2.78-2.55 (m, 4H, CH_2), 2.13-1.89 (m, 4H, CH_2); ^{13}C NMR (100MHz, d_6-DMSO): δ = 172.08, 171.99, 165.88, 152.45, 149.18, 141.95, 141.78, 136.59, 135.77, 132.48, 130.16, 129.53, 129.18, 129.05, 128.79, 127.50, 126.32, 123.85, 54.07, 52.96, 40.56, 34.06, 33.82, 32.38, 31.89; ESI-MS: m/z = 569.3 $[M+H]^+$.

4.2 Biological evaluation

4.2.1 *In vitro* 20S proteasome chymotrypsin-like inhibition assay

Chymotrypsin-like enzyme activity assay was carried out in 50 μL volume and all the assay components were diluted in Tris-HCl buffer (100 mM Tris-HCl, pH 8.0). The reaction was carried out in black 384-well plates. 1 μL compound was added into 10 μL purified human proteasome (25 μg/mL), a gift from Dr. Jiang-ping Wu (Notre-Dame Hospital, Montreal, Quebec, Canada), incubated for 15 min, and then added with 39 μL synthesized substrate Suc-Leu-Leu-Val-Tyr-AMC (50 μM, GL Biochem Ltd., Shanghai, P.R. China) as the reference reported. And the AMC of probe was detected by monitoring the increase of fluorescence with Envision, at 355 nm excitation and 460 nm Emission. The IC_{50} data was calculated using the software GraphPad Prism, and chosen the equation "sigmoidal dose-response (variable slope)" for curve fitting.

4.2.2 Tumor cell anti-proliferation assay

3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). Human multiple myeloma (MM) cell lines (RPMI 8226 and MM-1S) were purchased from Invitrogen (Grand Island, NY, USA), and were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin-Streptomycin from Invitrogen (Grand Island, NY, USA) at 37°C in a 5% CO_2 humidified atmosphere.

A 100 μL MM-1S (2×10^4/well) or RPMI 8226 cells (0.5×10^4/well) were seeded into 96-well plates. After treated with tested compounds for 72h, cells were added with MTS at a final concentration of 0.5 mg/mL for 2 to 4 hours. Optical density was determined at 490 nm (background subtraction at 690 nm) by SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The growth inhibitory ratio was calculated as follows: Growth inhibitory ratio = $(A_{control}-A_{sample})/ A_{control}$. IC_{50} values were derived from a nonlinear regression model (curvefit) based on sigmoidal dose response curve (variable slope) and computed using Graghpad Prism version 5.02 (Graphpad Software).

4.2.3 *Ex vivo* blood cell proteasome inhibition assay

Whole blood was collected from normal mice and compounds at appropriate concentrations were added (the volume ratio of the compound to the serum was 1:50). After incubation for 40 min, the above samples are centrifuged at 1,000 rpm for 5 min and the supernatants are removed. Blood cells were added 100 μL EDTA (5 mM, pH=8.0) and lysed at 4ºC for 60 minutes, then centrifuged at 6,600 ×g for 10 min and the supernatants were collected. Then the protein concentration of the supernatants was measured with Bradford method and determined the activity of the proteasome. The assays were carried out in a final volume of 50 μL containing 20 mmol/L HEPES, pH=8.0, 0.5 mmol/L EDTA, 0.05% SDS, 50 μM substrate (Suc-Leu-Leu-Val-Tyr-AMC), 1% DMSO, and blood cell protein 100 μg, and detected at 355 nm excitation and 460 nm Emission as the *in vitro* assay mentioned.

4.2.4 Enzymatic stability assay

Plasma or blood was prepared on sodium heparin. Stock solutions were prepared at 10 mM in DMSO for the test compounds. Aliquots of the stock solutions were diluted to 0.02 mM in 0.05 M sodium phosphate buffer containing 0.5% BSA as the dosing solution. Then 10 μL of the dosing solutions were dosed into 90 μL of pre-warmed plasma or blood (37°C) in duplicates (n=2) in 96-well assay plates to reach a final test concentration of 2 μM. The plates were kept in a 37°C water bath for the duration of the experiment. At each time point, 400 μL of acetonitrile was added into corresponding wells of the assay plates. After the final time point was quenched, the assay plates were ultrasonicated for 2 min,

shaken at the vibrator (IKA, MTS 2/4) for 10 min (600 rpm/min) and then centrifuged at 5594g for 15 min (Thermo Multifuge × 3R). Aliquots of the supernatant were taken, diluted 1:1 into distilled water, and analyzed by LC-MS/MS. The peak area response ratio to internal standard (PARR) of the compounds at different time point was compared to the PARR at time 0 to determine the percent of test compound remaining. Half-lives ($T_{1/2}$) were calculated using Excel software, fitting to a single-phase exponential decay equation.

Conflict of interest

The authors confirm that this article content has no conflict of interest.

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Figure captions:

Figure 1. Structures of the three approved proteasome inhibitors

Figure 2. Structures of representative non-covalent proteasome inhibitors

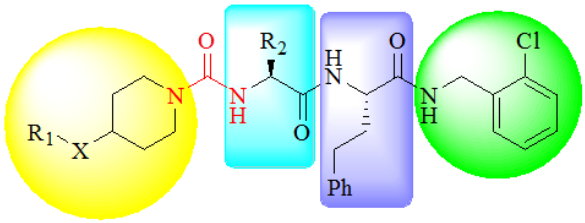
Figure 3. Non-peptide fragment (piperidine) constructed non-covalent proteasome inhibitors

Figure 4. *Ex vivo* blood cell proteasome inhibitory activities of selected compounds (**24** and **27-29**)

Scheme 1. Synthesis of piperidine-containing fragments (**9** and **10a-g**). Reagents and conditions: (I) HOBt, EDCI, 1-Boc-4-aminopiperidine, diisopropylethylamine, DCM, 0^{o}C-rt; (II) *N*-Boc-4-piperidinecarboxylic acid, $SOCl_2$, pyridine, Et_3N, DMAP, DCM, rt; (III) trifluoroacetic acid, DCM, 0^{o}C-rt.

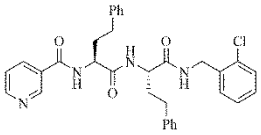
Scheme 2. Synthesis of piperidine-containing target compounds (**16-29**). Reagents and conditions: (I) Boc-L-hPhe, HOBt, EDCI, diisopropylethylamine, DCM, 0^{o}C-rt; (II) trifluoroacetic acid, DCM, 0^{o}C-rt; (III) corresponding Boc-protected amino acid, HOBt, EDCI, diisopropylethylamine, DCM, 0^{o}C-rt; (IV) triphosgene, diisopropylethylamine, DCM, aqueous $NaHCO_3$, 0^{o}C-rt.

Graphical abstract



Compound **28**:
R_1=pyridin-3-yl, X=NHCO, R_2=phenylethyl
IC_{50}= 1.4 nM (Proteasome CT-L)

Table **3**. Enzymatic stability of compound **28** and its corresponding dipeptidyl analogue without piperidine ring

Compound	Half-life ($T_{1/2}$, min)	
	plasma	blood
28	435.3	178.7
	285.3	111.0