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Research paper

Exploration of novel piperazine or piperidine constructed non-covalent peptidyl derivatives as proteasome inhibitors

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ABSTRACT

A series of novel piperazine or piperidine-containing non-covalent peptidyl derivatives possessing a neopentyl-asparagine residue were designed, synthesized and evaluated as proteasome inhibitors. All target compounds were screened for their 20S proteasome chymotrypsin-like inhibitory activities, and 15 ones displayed more potent activities than carfilzomib with IC_{50} values lower than 10 nM. Subsequently, the most potent 10 analogues were tested for their cytotoxic activities against two multiple myeloma (MM) cell lines RPMI-8226 and MM-1S. Based on these experiments, selected derivatives were further evaluated for their *ex vivo* and *in vivo* blood cell proteasome inhibitory activities. The most potential compound **35** (proteasome inhibition IC_{50}: 1.2 ± 0.1 nM) with potent anti-proliferation (IC_{50}: RPMI-8226 8.4 ± 0.8 nM; MM-1S: 6.3 ± 0.8 nM), *ex vivo* and *in vivo* activities also had a prolonged half life in plasma, which demonstrated that the enzymatic stabilities of this series of compounds have been improved by constructing a six-membered ring into the peptide skeleton. All the experiments confirmed the correctness of design concept, which made this series of compounds potential leads for exploring new anti-MM drugs.

1. Introduction

The ubiquitin-proteasome pathway (UPP) mediated protein degradation was discovered about 40 years ago, and three scientists Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize for Chemistry in 2004 for this important finding [1–3]. Several components such as ubiquitin, ubiquitination related enzymes (E1, E2 and E3), deubiquitinating enzyme (DUB) and proteasome were included in this crucial protein degradation pathway, which helped misfolded or damaged proteins to be recognized, translocated, unfolded and hydrolyzed in this cascade [4–8]. All of these components have been extensively studied and the prominent proteasome has been considered and validated as one of the most important drug targets [8–10]. To date, three proteasome inhibitors have been approved by the FDA for the treatment of multiple myeloma (MM) and mantle cell lymphoma: peptide boronates bortezomib and ixazomib together with peptide epoxyketone carfilzomib (Fig. 1A) [11–13]. Besides, several proteasome inhibitors are in different stages of clinical trials, including oprozomib (ONX-0912, Fig. 1B) and marizomib (NPI-0052, Fig. 1B) [4]. Actually, established proteasome inhibitors are also correlated with some other diseases including immunological [14], neurodegenerative [15], cardiovascular diseases [16], and even malaria [17].

Proteasome inhibitors can be classified into covalent and non-covalent types according to their different binding modes with the hydroxyl group of the *N*-terminal threonine residue of proteasome [4]. All of the three approved proteasome inhibitors are covalent ones with a Michael acceptor as an electrophilic moiety at the *C*-terminal. The firm covalent interaction with the receptor

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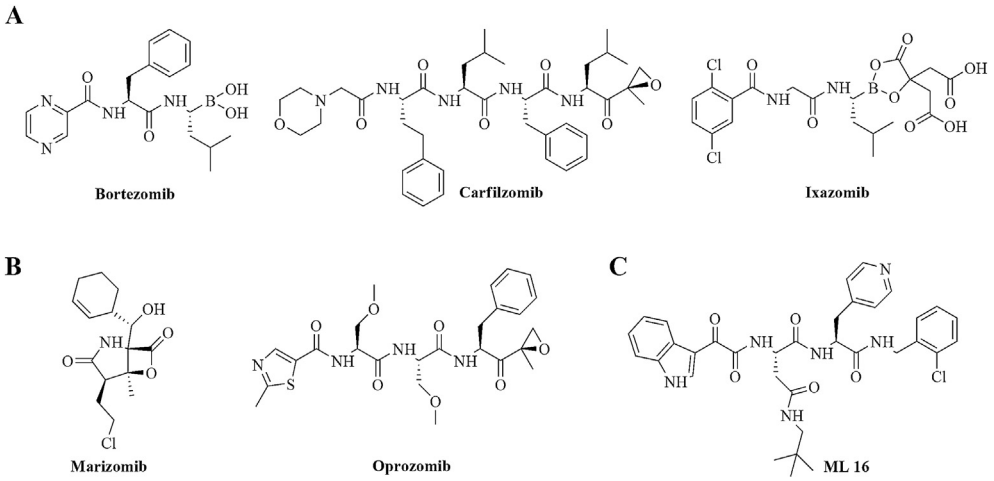


Fig. 1. **A**: FDA approved proteasome inhibitors for cancer therapy; **B**: Proteasome inhibitors in clinical trials; **C**: Representative of non-covalent proteasome inhibitors.

leads to a prolonged biological action, but may be not beneficial if the desired proteasome function is limited and induce severe side effects [18]. Besides, these excessively reactive inhibitors have very short half lives, which covalently combine with the blood cell proteasome quickly and fail to treat solid cancers [19]. In contrast, non-covalent proteasome inhibitors are less reactive, and their reversible interactions with proteasome ensure them with rapid binding and dissociation kinetics [19]. Therefore, more therapeutic advantages such as fewer side effects and broader usage for solid cancers are probably to be achieved. Nowadays, non-covalent proteasome inhibitors have attracted more and more attention, and some of these compounds displayed comparable or even better proteasome inhibitory activities [19–21]. Representative analogue ML 16 (Fig. 1C) was developed by Blackburn and colleagues from Millennium Pharmaceuticals, Inc., which was the most potent non-covalent inhibitor discovered so far with IC_{50} values of 1.2 nM and 1.1 nM against constitutive proteasome and immunoproteasome, respectively [19]. Further study of this series of compounds may offer opportunities for discovering new anti-cancer agents.

In an effort to identify novel non-covalent proteasome inhibitors with better pharmacokinetic profiles, a piperidine ring was introduced into the peptide skeleton in our previous research [22]. The target compounds exhibited better enzymatic stabilities both in plasma and blood together with good proteasome inhibitory activities. In this manuscript, a new series of piperazine or piperidine-containing non-covalent proteasome inhibitors with retained critical neopentylaminated aspartic acid of ML 16 were designed and synthesized (Fig. 2), and these compounds showed not only improved enzymatic stabilities, but also good *in vitro* and *in vivo* activities with the potential for further development.

2. Results and discussion

2.1. Chemistry

The synthetic routes for piperazine or piperidine-containing fragments **7a-b**, **8a-b**, **9a-b** and **10a-i** are summarized in Scheme 1. An easy condensation of corresponding acid **1a-b** with protected piperazine or piperidine fragment provided compounds **3a-b**, **4a-b** and **5a-b**. However, due to low reactive activities of various arylamine, compounds **6a-i** were synthesized from arylamine **2a-i** and *N*-Boc-4-piperidineformyl chloride. Afterwards, deprotection of **3a-b**, **5a-b** and **6a-i** with trifluoroacetic acid (TFA) and **4a-b** with LiOH afforded piperazine or piperidine TFA salts (**7a-b**, **9a-b** and **10a-i**) and piperidinecarboxylic acids (**8a-b**).

Dipeptide fragments were synthesized following the method described in Scheme 2. Reaction of Boc-L-aspartic acid-1-benzyl ester (**11**) with neopentylamine furnished compound **12**, which was deprotected at the presence of Pd/C (10%) to afford compound **13**. **16a-f** were synthesized from Boc-protected amino acid **14a-d** with condensation and deprotection, which were reacted with fragment **13** and deprotected again to provide the dipeptide TFA salts **18a-f**. Subsequently, the target compounds **19-43** were synthesized with the methods illustrated in Schemes 3 and 4. The primary amine **18a-f** were either condensed with **8a-b** to afford **19-20**, or transformed to corresponding isocyanate intermediates and

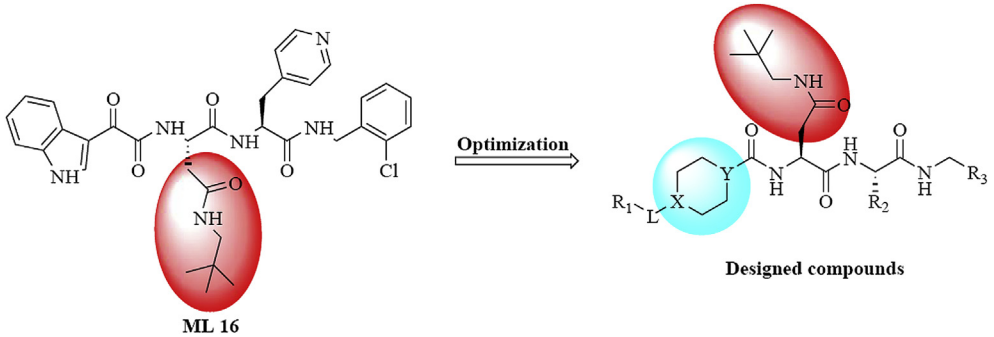
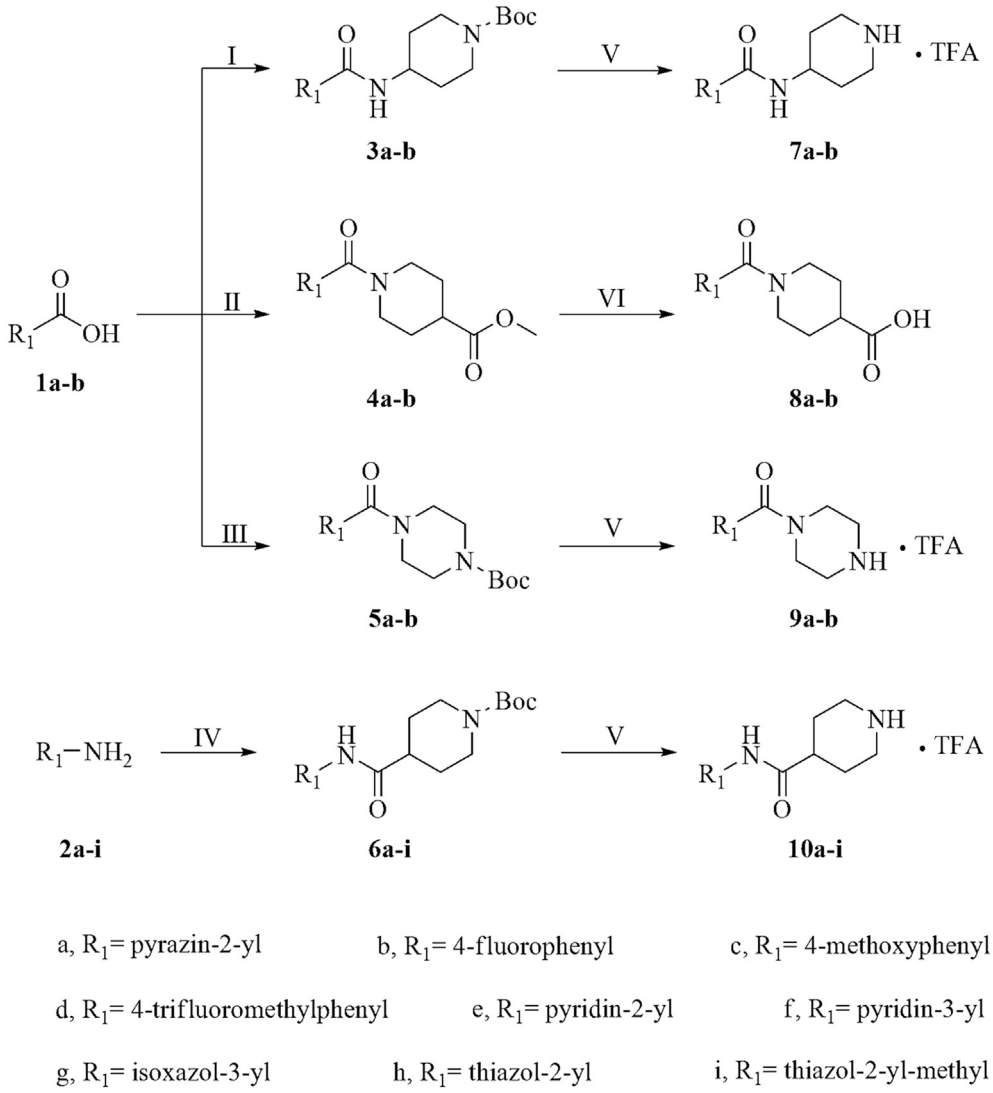


Fig. 2. Design strategy of the piperazine or piperidine-containing non-covalent proteasome inhibitors.



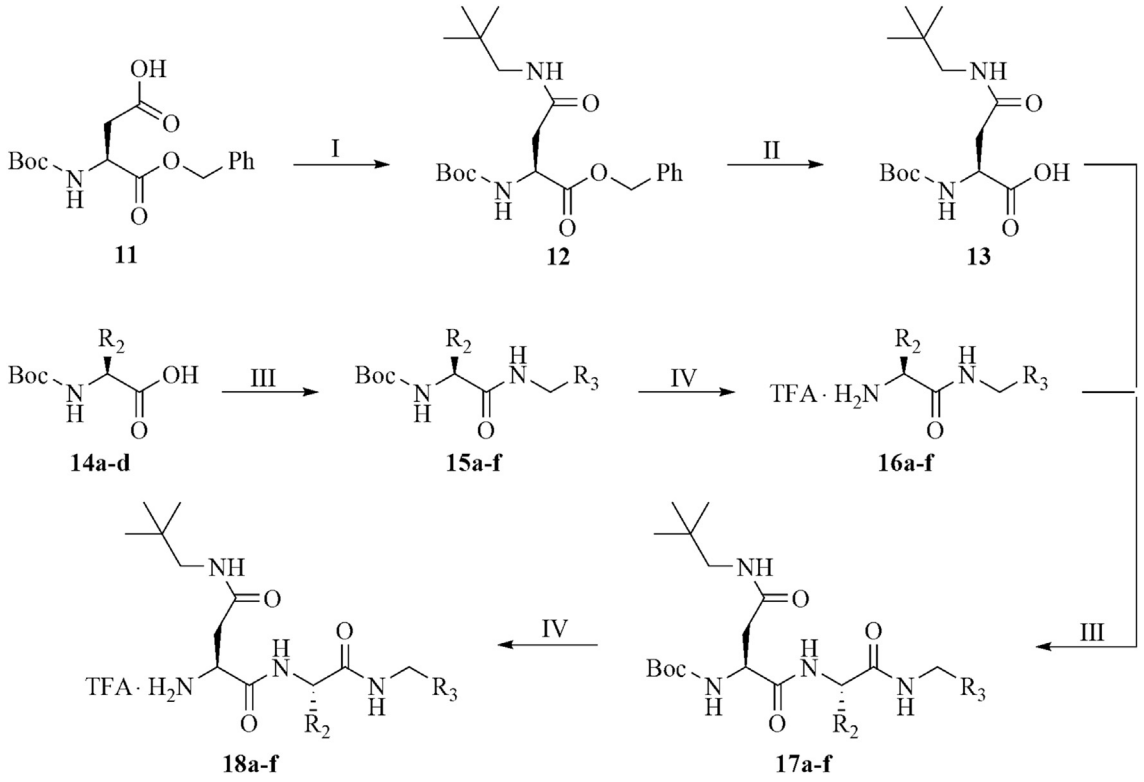
Scheme 1. Synthesis of piperazine or piperidine-containing fragments (**7a-b**, **8a-b**, **9a-b** and **10a-i**). Reagents and conditions: (I) HOBt, EDCI, *N*-Boc-4-aminopiperidine, diisopropylethylamine (DIPEA), DCM, 0°C-rt; (II) HOBt, EDCI, methyl piperidine-4-carboxylate, DIPEA, DCM, 0°C-rt; (III) HOBt, EDCI, *N*-Boc-piperazine, DIPEA, DCM, 0°C-rt; (IV) *N*-Boc-4-piperidinecarboxylic acid, $SOCl_2$, pyridine, Et_3N, DMAP, DCM, rt; (V) TFA, DCM, 0°C-rt; (VI) LiOH, H_2O, acetone, rt.

reacted with **7a-b**, **9a-b** and **10a-i** to obtain target compounds **21-43**.

2.2. Proteasome inhibitory activities

All 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, almost all the target compounds showed potent proteasome chymotrypsin-like inhibitory activities, and even 15 compounds were more potent than the positive control carfilzomib with IC_{50} values lower than 10 nM. Various R_2 replacements influenced the proteasome inhibitory activities significantly. Phenylethyl substituted analogues were 10–40-fold more potent than methyl, iso-butyl and benzyl substituted compounds (**29** versus **23**, **25** and **27**; **30** versus **24**, **26** and **28**). Therefore, phenylethyl was employed at R_2 group for further optimization. In order to evaluate the influences of R_3, two more substituents were replaced. Although compounds **31-34** showed potent proteasome inhibitory activities with IC_{50} values ranging from 2.9 to 10.8 nM, they were less potent than the corresponding 2-chlorophenyl replaced analogues (IC_{50}: **29**, 1.1 ± 0.1 nM; **30**, 0.6 ± 0.1 nM). Different 6-membered rings introduced into the skeleton of the peptide seemed to have little impact on the inhibitory activity. Analogues **21** and **22** constructed form piperazine also exhibited significant activities with IC_{50} values of 5.5 ± 0.2 nM and 3.8 ± 0.5 nM, respectively. The linker L between the 6-membered ring and the substituents R_1 was also crucial for the activity. Generally speaking, NHCO was more tolerable than CONH as a linker. Most NHCO substituted compounds were more potent than the corresponding CONH substituted analogues except two pairs of compounds (**23** versus **24**; **35** versus **36**). Additionally, further study of impact of the N-terminus group (R_1) on activity was performed. Among these compounds (**30** and **36-43**), most were more potent than the positive control carfilzomib, and different R_1 replacements seemed to have little differences. However, compounds with 4-methoxyphenyl and 4-trifluoromethylphenyl displayed weak activities with IC_{50} values of 25.7 and 192.5 nM, respectively. Causes of this discrepancy need



Scheme 2. Synthesis of dipeptide fragments **18a-f**. Reagents and conditions: (I) HOBt, EDCI, neopentylamine hydrochloride, DIPEA, DCM, 0°C-rt; (II) Pd/C (10%), H_2, MeOH, rt; (III) HOBt, EDCI, DIPEA, DCM, 0°C-rt; (IV) TFA, DCM, 0°C-rt.

to be further studied.

2.3. Tumor cell growth inhibitory activities

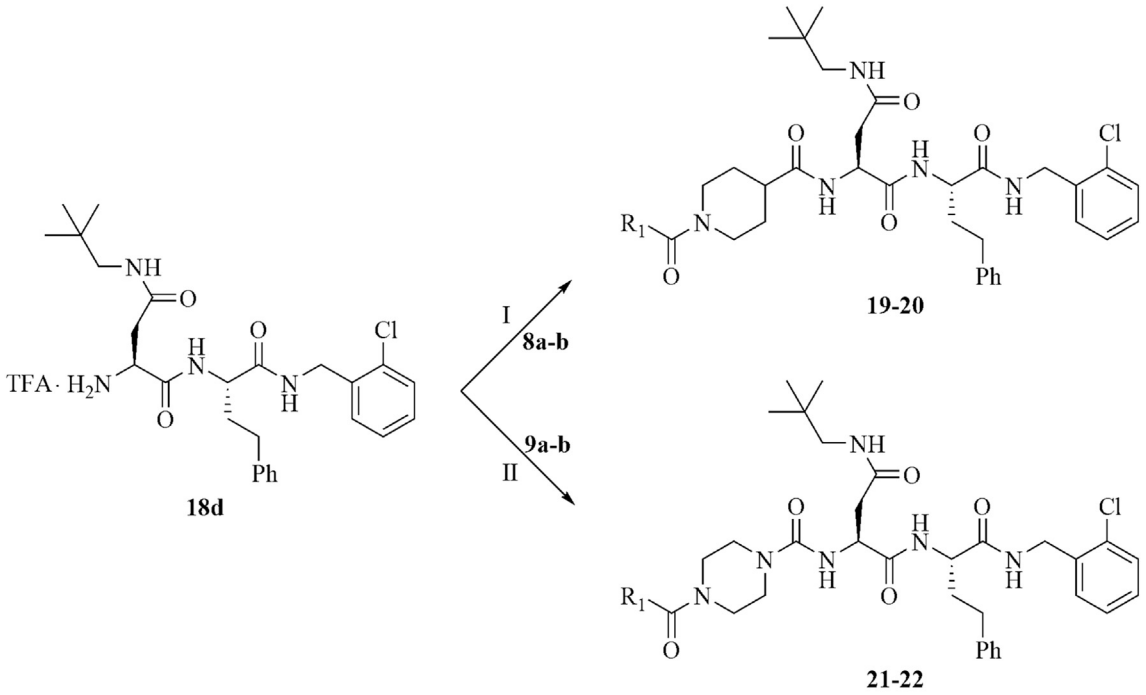
Based on the evaluation of proteasome chymotrypsin-like inhibitory activities, 10 selected compounds (**19-20**, **29-30**, **35-36**, **39-40** and **42-43**) with IC_{50} values lower than 3 nM were further tested for their tumor cell (RPMI-8226 and MM-1S) growth inhibitory activities *in vitro* by MTS assay with carfilzomib employed as the positive control. According to the results summarized in Table 2, all the tested compounds displayed potent cytotoxic activities, which was consistent with the proteasome inhibitory activities. Among them, **40** displayed the most potent anti-proliferative activities against two MM cell lines RPMI-8226 and MM-1S with IC_{50} values of 3.8 ± 0.5 nM and 4.3 ± 0.7 nM, respectively.

2.4. Ex vivo blood cell proteasome inhibitory activities

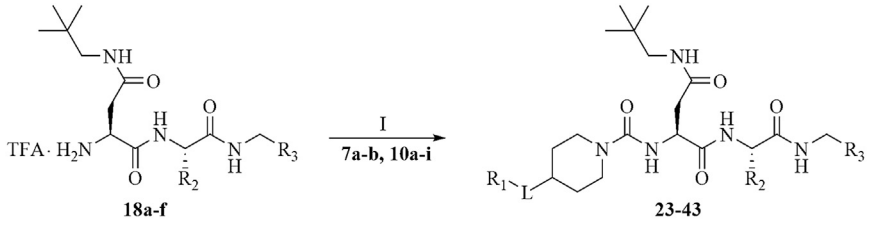
Subsequently, nine compounds were selected and further screened for the blood cell proteasome inhibitory activities *ex vivo* with compound **27** employed as the negative control and carfilzomib as the positive control. As illustrated in Fig. 3 and analogues (**36**, **42** and **43**) showed comparable blood cell proteasome inhibitory activities compared to that of the positive control carfilzomib at both concentrations of 0.5 μM and 2.5 μM. Besides, another 3 compounds (**30**, **35** and **39**) displayed more potent activities with inhibitory rates of more than 70% at the concentration of 2.5 μM, and compound **35** was the most potent one. After incubation in the whole blood and various enzyme metabolisms, these compounds still displayed potent activities, which suggested that the stabilities of the analogues have been improved by constructing 6-membered ring into the peptide skeleton.

2.5. In vivo blood cell proteasome inhibitory activities

The most potent compound **35** in the *ex vivo* assay was further tested for its *in vivo* blood cell proteasome inhibitory activities to verify whether this analogue has any potential for clinical application. Carfilzomib was employed as the positive control. As illustrated in Fig. 4, compound **35** displayed the most potent proteasome inhibitory activity with inhibitory rate of more than 50% 2 h after dosing at the dosage of 5 mg/kg. The extent of proteasome inhibition induced by compound **35** was a process of firstly enhancing and then weakening, which was different from that of the inhibition induced by carfilzomib and demonstrated the non-covalent reversible interaction between this series of compounds and proteasome. Compared to carfilzomib, the *in vivo* activity of compound **35** was less potent, which needs to be further optimized.



Scheme 3. Synthesis of piperazine or piperidine-containing target compounds (**19-22**). Reagents and conditions: (I) HOBt, EDCI, DIPEA, DCM, 0°C-rt; (II) triphosgene, DIPEA, DCM, aqueous $NaHCO_3$, 0°C-rt.



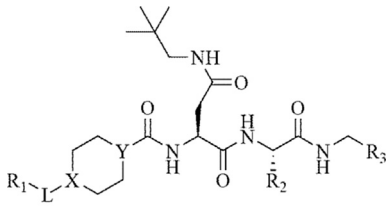
	R_1	L	R_2	R_3		R_1	L	R_2	R_3
23	pyrazin-2-yl	CONH	methyl	2-chlorophenyl	**24**	pyrazin-2-yl	NHCO	methyl	2-chlorophenyl
25	pyrazin-2-yl	CONH	iso-butyl	2-chlorophenyl	**26**	pyrazin-2-yl	NHCO	iso-butyl	2-chlorophenyl
27	pyrazin-2-yl	CONH	benzyl	2-chlorophenyl	**28**	pyrazin-2-yl	NHCO	benzyl	2-chlorophenyl
29	pyrazin-2-yl	CONH	phenylethyl	2-chlorophenyl	**30**	pyrazin-2-yl	NHCO	phenylethyl	2-chlorophenyl
31	pyrazin-2-yl	CONH	phenylethyl	2, 4-difluorophenyl	**32**	pyrazin-2-yl	NHCO	phenylethyl	2,4-difluorophenyl
33	pyrazin-2-yl	CONH	phenylethyl	benzo[d][1,3]dioxol-5-yl	**34**	pyrazin-2-yl	NHCO	phenylethyl	benzo[d][1,3]dioxol-5-yl
35	4-fluorophenyl	CONH	phenylethyl	2-chlorophenyl	**36**	4-fluorophenyl	NHCO	phenylethyl	2-chlorophenyl
37	4-methoxyphenyl	NHCO	phenylethyl	2-chlorophenyl	**38**	4-trifluoromethylphenyl	NHCO	phenylethyl	2-chlorophenyl
39	pyridin-3-yl	NHCO	phenylethyl	2-chlorophenyl	**40**	pyridin-2-yl	NHCO	phenylethyl	2-chlorophenyl
41	isoxazol-3-yl	NHCO	phenylethyl	2-chlorophenyl	**42**	thiazol-2-yl	NHCO	phenylethyl	2-chlorophenyl
43	thiazol-2-yl-methyl	NHCO	phenylethyl	2-chlorophenyl					

Scheme 4. Synthesis of piperidine-containing target compounds (**23-43**). Reagents and conditions: (I) triphosgene, DIPEA, DCM, aqueous $NaHCO_3$, 0°C-rt.

2.6. Plasma stability

Based on the *in vitro*, *ex vivo* and *in vivo* assays against proteasome, selected compound **35** and its corresponding dipeptidyl analogue without piperidine ring (4-fluorophenyl-carbonyl- Asp (NH-neopentyl)-hPhe-NH-2-chlorobenzyl) were tested for their enzymatic stability in mouse plasma. As summarized in Table 3, the half life of compound **35** (672.4 min) was 2-fold longer than the compound without six-membered ring (336.4 min), which demonstrated the enzymatic stability of this series of compounds have been increased by constructing the six-membered ring into the peptide skeleton.

Table 1
20S proteasome chymotrypsin-like inhibitory activities of target compounds (**19-43**).



19-43

Compd.	R_1	L	X/Y	R_2	R_3	IC_{50} (nM)[a]
19	pyrazin-2-yl	CO	N/C	phenylethyl	2-chlorophenyl	2.4 ± 0.1
20	4-fluorophenyl	CO	N/C	phenylethyl	2-chlorophenyl	2.6 ± 0.1
21	pyrazin-2-yl	CO	N/N	phenylethyl	2-chlorophenyl	5.5 ± 0.2
22	4-fluorophenyl	CO	N/N	phenylethyl	2-chlorophenyl	3.8 ± 0.5
23	pyrazin-2-yl	CONH	C/N	methyl	2-chlorophenyl	13.3 ± 3.7
24	pyrazin-2-yl	NHCO	C/N	methyl	2-chlorophenyl	19.7 ± 3.9
25	pyrazin-2-yl	CONH	C/N	iso-butyl	2-chlorophenyl	21.8 ± 0.7
26	pyrazin-2-yl	NHCO	C/N	iso-butyl	2-chlorophenyl	11.6 ± 0.4
27	pyrazin-2-yl	CONH	C/N	benzyl	2-chlorophenyl	41.0 ± 6.9
28	pyrazin-2-yl	NHCO	C/N	benzyl	2-chlorophenyl	18.0 ± 1.4
29	pyrazin-2-yl	CONH	C/N	phenylethyl	2-chlorophenyl	1.1 ± 0.1
30	pyrazin-2-yl	NHCO	C/N	phenylethyl	2-chlorophenyl	0.6 ± 0.1
31	pyrazin-2-yl	CONH	C/N	phenylethyl	2, 4-difluorophenyl	10.8 ± 0.6
32	pyrazin-2-yl	NHCO	C/N	phenylethyl	2, 4-difluorophenyl	6.6 ± 1.1
33	pyrazin-2-yl	CONH	C/N	phenylethyl	benzo[d][1,3]dioxol-5-yl	10.0 ± 0.8
34	pyrazin-2-yl	NHCO	C/N	phenylethyl	benzo[d][1,3]dioxol-5-yl	2.9 ± 0.5
35	4-fluorophenyl	CONH	C/N	phenylethyl	2-chlorophenyl	1.2 ± 0.1
36	4-fluorophenyl	NHCO	C/N	phenylethyl	2-chlorophenyl	1.5 ± 0.3
37	4-methoxyphenyl	NHCO	C/N	phenylethyl	2-chlorophenyl	25.7 ± 1.8
38	4-trifluoromethyl-phenyl	NHCO	C/N	phenylethyl	2-chlorophenyl	192.5 ± 18.3
39	pyridin-3-yl	NHCO	C/N	phenylethyl	2-chlorophenyl	0.7 ± 0.1
40	pyridin-2-yl	NHCO	C/N	phenylethyl	2-chlorophenyl	1.0 ± 0.1
41	isoxazol-3-yl	NHCO	C/N	phenylethyl	2-chlorophenyl	7.0 ± 1.3
42	thiazol-2-yl	NHCO	C/N	phenylethyl	2-chlorophenyl	0.9 ± 0.2
43	thiazol-2-yl-methyl	NHCO	C/N	phenylethyl	2-chlorophenyl	1.0 ± 0.1
Carfilzomib	–	–	–	–	–	8.4 ± 0.9

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

Table 2
Cytotoxic activities of selected compounds against two MM cell lines.

Compound	Cytotoxicity (IC_{50}, nM)[a]	
	RPMI 8226	MM-1S
19	15.9 ± 2.4	11.8 ± 0.7
20	22.7 ± 3.1	31.8 ± 5.4
29	20.3 ± 2.7	18.8 ± 2.6
30	17.1 ± 1.5	15.8 ± 2.9
35	8.4 ± 0.8	6.3 ± 0.8
36	13.9 ± 2.4	12.5 ± 3.7
39	20.8 ± 3.8	20.7 ± 1.4
40	3.8 ± 0.5	4.3 ± 0.7
42	10.2 ± 1.6	7.2 ± 1.1
43	20.7 ± 4.1	22.5 ± 3.6
Carfilzomib	13.2 ± 0.6	1.5 ± 0.6

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

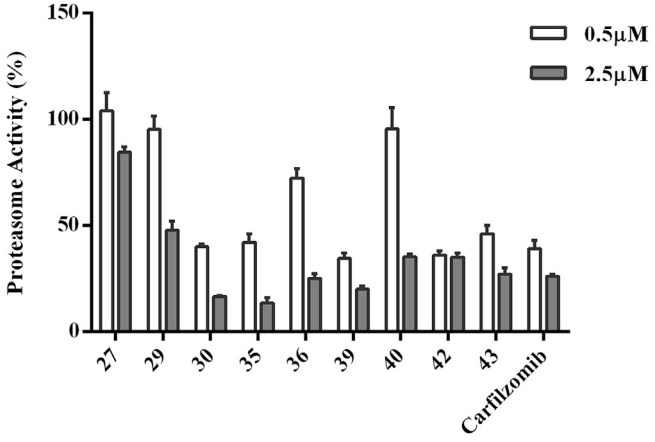


Fig. 3. *Ex vivo* blood cell proteasome inhibitory activities of selected compounds.

2.7. Binding mode analysis

To explore the binding modes of target compound with the active site of proteasome, molecular docking simulation studies were carried out by using Glide module of Schrödinger package. The co-crystal structure of proteasome (PDB ID code: 3MG6) was selected as the docking template. The binding modes between proteasome and the most potent compound **30** were shown in Fig. 5. As depicted in Fig. 5, the *C*-terminal 2-chlorobenzyl group and the neopentylamino group occupied well defined S1 and S3 pockets, respectively, while the homo-phenylalanine residue was oriented toward the solvent. Besides, some critical Hydrogen bonds were formed between the carbonyl and amino groups of the peptide skeleton and Gly47, Thr21, Ala49, Ala50 and Asp114 of the proteasome active site. These hydrogen bonds played important roles in the inhibitory potency against proteasome. For the inactive

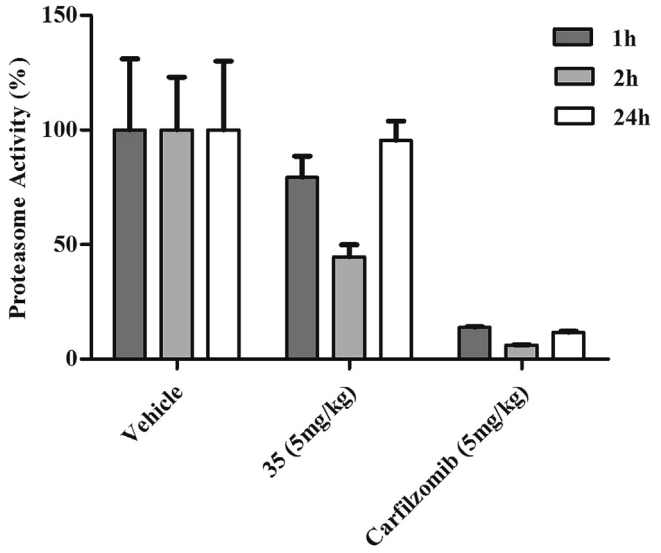
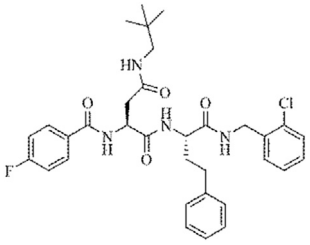


Fig. 4. *In vivo* blood cell proteasome inhibitory activities of compound **35.**

Table 3
Plasma half-lives of compound **35** and its corresponding dipeptidyl analogue without piperazine or piperidine ring.

Compound	Plasma Half-life ($t_{1/2}$, min)
35 	672.4 336.4

3. Conclusion

A series of novel piperazine or piperidine-containing non-covalent peptidyl derivatives possessing a neopentyl-asparagine residue were synthesized and evaluated for their proteasome inhibitory activities. Among all the synthesized compounds, 15 analogues exhibited proteasome inhibitory activities more potent than the positive control carfilzomib, which also possessed forceful anti-proliferation activities against two MM cell lines RPMI-8226 and MM-1S. In addition, 6 compounds displayed comparable or even more potent activities against proteasome in the *ex vivo* blood cell assay. Besides, the most potent compound **35** showed moderate and reversible blood cell proteasome inhibitory activity in the *in vivo* assay. The improved enzymatic stability of this series of compounds could be validated by the 2-fold longer half life of compound **35** than its corresponding dipeptidyl analogue without piperidine ring. Compound **35** should be considered to be further studied as a lead and deserves additional optimization.

4. Experimental procedures

4.1. Chemistry

^{1}H and ^{13}C NMR spectra were recorded on Brüker 500/400 MHz 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.

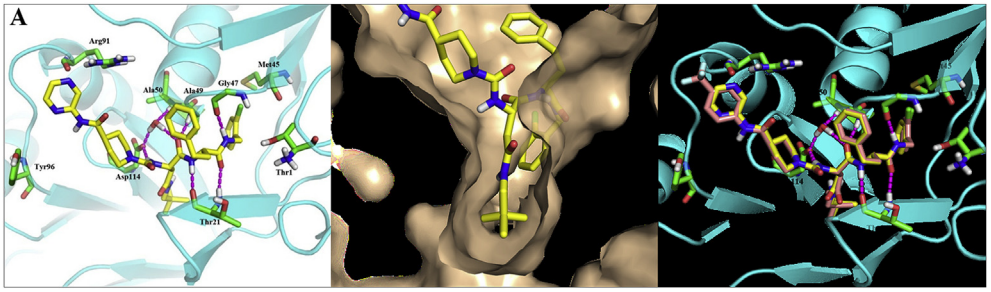


Fig. 5. The binding mode of selected compound with chymotrypsin-like active site of 20S proteasome (PDB ID: 3MG6). **A**: Predicted H-bonds between compound **30** and the protein, the H-bonds are shown as dashed magenta lines; **B**: Occupancy of the S3 binding pocket of the 20S proteasome by the neopentyl-asparagine residue of compound **30**; **C**: Overlay of the most potent compound **30** with the inactive compound **38**, and the H-bonds are shown as dashed magenta lines.

compound **38**, the modeling results indicated that it could bind to proteasome with a similar binding mode of the most active compound **30** (Fig. 5C). The activity difference may arise from different properties of the two analogues. The electronegativity of N atom of pyrazine may interact with the nearby Arg91 with positive charge. Besides, both pyrazine and trifluoromethylphenyl group located in a solvent exposed area. Pyrazine (CLogP: −0.312) may be more favorable than the highly hydrophobic trifluoromethylphenyl (CLogP: 3.025) in this area. The molecular docking study may allow the rational design of more potent proteasome inhibitors.

4.1.1. General procedure for the synthesis of piperidine or piperazine-containing fragments (***3a-b***, ***4a-b*** and ***5a-b***)

To a reaction of corresponding carboxylic acid (**1a-b**, 10.0 mmol) in DCM (40.0 mL), HOBt (1.49 g, 11.0 mmol) and EDCI (2.88 g, 15.0 mmol) were added at 0 °C. The mixture was kept at the same temperature and was stirred for 30 min. Then corresponding piperidine or piperazine fragment (10.0 mmol) and

diisopropylethylamine (3.7 mL, 20.0 mmol) were added. After stirring at room temperature for 3 h, the resulting mixture was washed with aqueous $NaHCO_3$ solution (1 × 30 mL), brine (1 × 30 mL) and dried over Na_2SO_4. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography (ethyl acetate: petroleum ether = 2:1–1:1). Compound **3a** has been reported in our previous work.

*4.1.1.1. tert-Butyl 4-(4-fluorobenzamido) piperidine-1-carboxylate (**3b**).* White solid; Yield: 92%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.78 (dd, 2H, J = 8.5, 5.0 Hz, Ar-H), 7.11 (t, 2H, J = 8.5 Hz, Ar-H), 6.08 (d, 1H, J = 7.5 Hz, NH), 4.15–4.08 (m, 3H, CH + CH_2), 2.89 (m, 2H, CH_2), 2.01 (d, 2H, J = 11.0 Hz, CH_2), 1.46 (s, 9H, CH_3), 1.43–1.39 (m, 2H, CH_2); ESI-MS: m/z = 323.2 $[M+H]^+$.

*4.1.1.2. Methyl 1-(pyrazine-2-carbonyl)piperidine-4-carboxylate (**4a**).* White solid; Yield: 94%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.88 (d, 1H, J = 1.0 Hz, pyrazine-H), 8.61 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.52 (s, 1H, pyrazine-H), 4.51 (m, 1H, CH_2), 3.91 (m, 1H, CH_2), 3.70 (s, 3H, CH_3), 3.20 (m, 1H, CH_2), 3.09 (m, 1H, CH_2), 2.62 (m, 1H, CH), 2.04 (m, 1H, CH_2), 1.91 (m, 1H, CH_2), 1.81 (m, 2H, CH_2); ESI-MS: m/z = 250.1 $[M+H]^+$.

*4.1.1.3. Methyl 1-(4-fluorobenzoyl)piperidine-4-carboxylate (**4b**).* White solid; Yield: 87%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41 (dd, 2H, J = 8.5, 5.5 Hz, Ar-H), 7.10 (t, 2H, J = 8.5 Hz, Ar-H), 4.48 (m, 1H, CH_2), 3.80 (m, 1H, CH_2), 3.72 (s, 3H, CH_3), 3.06 (m, 2H, CH_2), 2.61 (m, 1H, CH), 1.96 (m, 2H, CH_2), 1.74 (m, 2H, CH_2); ESI-MS: m/z = 266.1 $[M+H]^+$.

*4.1.1.4. tert-Butyl 4-(pyrazine-2-carbonyl)piperazine-1-carboxylate (**5a**).* White solid; Yield: 76%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.97 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.65 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.54 (s, 1H, pyrazine-H), 3.79 (t, 2H, J = 5.0 Hz, CH_2), 3.62 (t, 2H, J = 5.0 Hz, CH_2), 3.56 (t, 2H, J = 5.0 Hz, CH_2), 3.49 (t, 2H, J = 5.0 Hz, CH_2), 1.47 (s, 9H, CH_3); ESI-MS: m/z = 293.2 $[M+H]^+$.

*4.1.1.5. tert-Butyl 4-(4-fluorobenzoyl)piperazine-1-carboxylate (**5b**).* White solid; Yield: 97%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.43 (dd, 2H, J = 9.0, 5.5 Hz, Ar-H), 7.12 (t, 2H, J = 8.5 Hz, CH_2), 3.54–3.41 (m, 8H, CH_2), 1.48 (s, 9H, CH_3); ESI-MS: m/z = 309.2 $[M+H]^+$.

*4.1.2. General procedure for the synthesis of piperidine-containing fragments (**6a-i**)*

To a mixture of *N*-Boc-4-piperidinecarboxylic acid (3.65 g, 16.0 mmol), pyridine (3.4 mL, 40.0 mmol) and CH_2Cl_2 (30.0 mL), $SOCl_2$ (1.5 mL, 19.0 mmol) was added under N_2 at room temperature. The mixture was stirred for half an hour, and a solution of corresponding amine (**2a-i**, 18.0 mmol), Et_3N (8.0 mL, 56.0 mmol) and a catalytic amount of DMAP in CH_2Cl_2 (30.0 mL) was added dropwise. The suspension was stirred overnight. The organic phase was washed with 1 N HCl (2 × 30 mL) and aqueous $NaHCO_3$ (2 × 30 mL), dried over Na_2SO_4 (For compounds **6a**, **6e** and **6f**, 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 **6a-i**. The details of compounds **6a-c**, **6e-f** and **6h** have been reported in our previous work.

*4.1.2.1. tert-Butyl 4-(4-(trifluoromethyl)phenylcarbamoyl)piperidine-1-carboxylate (**6d**).* Pale yellow oil; Yield: 60%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.09 (s, 1H, NH), 7.51–7.46 (m, 4H, Ar-H), 4.19–4.16 (m, 2H, CH_2), 2.81–2.72 (m, 2H, CH_2), 2.42–2.39 (m, 1H, CH), 1.88–1.84 (m, 2H, CH_2), 1.75–1.65 (m, 2H, CH_2), 1.48 (s, 9H, CH_3); ESI-MS: m/z = 373.2 $[M+H]^+$.

*4.1.2.2. tert-Butyl 4-(isoxazol-3-ylcarbamoyl)piperidine-1-carboxylate (**6g**).* White solid; Yield: 84%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 9.92 (s, 1H, NH), 8.35 (d, 1H, J = 2.0 Hz, isoxazole-H), 7.24 (d, 1H, J = 2.0 Hz, isoxazole-H), 4.14–4.08 (m, 2H, CH_2), 2.91–2.85 (m, 2H, CH_2), 2.64–2.59 (m, 1H, CH), 1.99 (d, 2H, J = 11.0 Hz, CH_2), 1.86–1.79 (m, 2H, CH_2), 1.47 (s, 9H, CH_3); ESI-MS: m/z = 296.2 $[M+H]^+$.

*4.1.2.3. tert-Butyl 4-(thiazol-2-ylmethylcarbamoyl)piperidine-1-carboxylate (**6i**).* White form; Yield: 87%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.71 (d, 1H, J = 3.0 Hz, thiazol-H), 7.30 (d, 1H, J = 3.0 Hz, thiazol-H), 6.58 (t, 1H, J = 4.5 Hz, NH), 4.77 (d, 2H, J = 5.5 Hz, CH_2), 4.23–4.04 (m, 2H, CH_2), 2.82–2.64 (m, 2H, CH_2), 2.37–2.31 (m, 1H, CH), 1.85 (d, 2H, J = 11.0 Hz, CH_2), 1.72–1.64 (m, 2H, CH_2), 1.46 (s, 9H, CH_3); ESI-MS: m/z = 326.3 $[M+H]^+$.

*4.1.3. General procedure for the synthesis of compounds **7a-b**, **9a-b** and **10a-i***

To a suspension of protected piperidine or piperazine-containing fragments **3a-b**, **5a-b** or **6a-i** (5.0 mmol) in CH_2Cl_2 (20.0 mL) was added trifluoroacetic acid (TFA, 5.0 mL) at 0 °C. The reaction mixture was then warmed to room temperature and stirred for 1 h. The volatiles were evaporated under reduced pressure and ether (30.0 mL) was added. White solid was precipitated, filtrated and dried, which was put into next step without further purification.

*4.1.4. Synthesis of piperidine-4-carboxylate analogues **8a-b***

To a solution of compounds **4a-b** (3.0 mmol) in acetone (10.0 mL), 6.0 mL 1 N LiOH (aq) was added dropwise. The mixture was stirred for 1 h and acetone was evaporated in vacuo. The residue was acidified to pH=2 with 1 N HCl and extracted with ethyl acetate (3 × 10 mL). The combined organic layers were washed with brine (1 × 10 mL), dried over Na_2SO_4, filtered and concentrated under reduced pressure. The crude products **8a-b** were put into next step without further purification.

*4.1.5. (S)-benzyl 2-(tert-butoxycarbonylamino)-4-(neopentylamino)-4-oxobutanoate (**12**)*

To a suspension of Boc-L-aspartic acid 1-benzyl ester (**11**, 3.23 g, 10.0 mmol) in CH_2Cl_2 (40.0 mL), HOBt (1.49 g, 11.0 mmol) and EDCI (2.88 g, 15.0 mmol) were added at 0 °C. The reaction mixture was kept at 0 °C and was stirred for 30 min. Then neopentylamine hydrochloride (1.24 g, 10.0 mmol) and diisopropylethylamine (3.7 mL, 20.0 mmol) were added at 0 °C. After stirring at room temperature for another 3 h, the resulting mixture was washed with aqueous $NaHCO_3$ solution (1 × 30 mL), brine (1 × 30 mL) and dried over Na_2SO_4. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography (ethyl acetate: petroleum ether = 1:3).

White solid; Yield: 99%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.41–7.30 (m, 5H, Ar-H), 5.80 (d, 1H, J = 6.5 Hz, NH), 5.66 (brs, 1H, NH), 5.17 (d, 2H, J = 5.0 Hz, CH_2), 4.55–4.52 (m, 1H, CH), 3.01 (d, 2H, J = 7.5 Hz, CH_2), 2.93 (dd, 1H, J = 18.0, 5.0 Hz, CH_2), 2.73 (dd, 1H, J = 19.0, 5.0 Hz, CH_2), 1.42 (s, 9H, CH_3), 0.87 (s, 9H, CH_3); ESI-MS: m/z = 393.3 $[M+H]^+$.

*4.1.6. (S)-2-(tert-butoxycarbonylamino)-4-(neopentylamino)-4-oxobutanoic acid (**13**)*

Compound **12** (1.96 g, 5.0 mmol) was dissolved in methanol (10.0 mL) in the presence of 10% palladium on carbon (10 mol%), and the reaction mixture was stirred under an atmosphere of H_2 for 2 h. The Pd/C was removed via filtration through celite, and the

solvent was evaporated to obtain compound **13** in quantitative yield. The crude product was used directly in the next step.

*4.1.7. General procedure for the synthesis of C-caped amino acids **15a-f***

To a suspension of corresponding *N*-Boc-protected amino acid (**14a-d**, 5.0 mmol) in DCM (20.0 mL), HOBt (0.75 g, 5.5 mmol) and EDCI (1.44 g, 7.5 mmol) were added. The reaction mixture was stirred for 30 min. Then corresponding amine (5.0 mmol) and diisopropylethylamine (1.9 mL, 10.0 mmol) were added and stirred for another 3 h. The resulting mixture was washed with aqueous $NaHCO_3$ solution (1 × 15 mL), brine (1 × 15 mL) and dried over Na_2SO_4. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography (ethyl acetate: petroleum ether = 1:5–1:2).

*4.1.7.1. Boc-Ala-NH-2-chlorobenzyl (**15a**).* White solid; Yield: 94%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.38–7.36 (m, 2H, Ar-H), 7.25–7.22 (m, 2H, Ar-H), 6.75 (brs, 1H, NH), 5.05 (brs, 1H, NH), 4.54 (d, 2H, J = 5.0 Hz, CH_2), 4.25–4.18 (m, 1H, CH), 1.43 (s, 9H, CH_3), 1.38 (d, 3H, J = 7.0 Hz, CH_3); ESI-MS: m/z = 313.1 $[M+H]^+$.

*4.1.7.2. Boc-Leu-NH-2-chlorobenzyl (**15b**).* White solid; Yield: 65%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.39–7.36 (m, 2H, Ar-H), 7.26–7.21 (m, 2H, Ar-H), 6.70 (t, 1H, J = 5.0 Hz, NH), 4.93 (d, 1H, J = 7.5 Hz, NH), 4.55 (t, 2H, J = 6.0 Hz, CH_2), 4.18–4.12 (m, 1H, CH), 1.76–1.66 (m, 2H, CH_2), 1.54-4-1.47 (m, 1H, CH), 1.46 (s, 9H, CH_3), 0.94 (t, 6H, J = 7.0 Hz, CH_3); ESI-MS: m/z = 355.2 $[M+H]^+$.

*4.1.7.3. Boc-Phe-NH-2-chlorobenzyl (**15c**).* White solid; Yield: 95%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.31 (d, 1H, J = 7.5 Hz, Ar-H), 7.27–7.10 (m, 8H, Ar-H), 6.25 (brs, 1H, NH), 5.07 (brs, 1H, NH), 4.44 (d, 2H, J = 6.0 Hz, CH_2), 4.38–4.33 (m, 1H, CH), 3.15–3.02 (m, 2H, CH_2), 1.40 (s, 9H, CH_3); ESI-MS: m/z = 389.2 $[M+H]^+$.

*4.1.7.4. Boc-hPhe-NH-2, 4-difluorobenzyl (**15e**).* White solid; Yield: 84%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.34–7.29 (m, 3H, Ar-H), 7.23–7.18 (m, 1H, Ar-H), 7.16 (d, 2H, J = 7.5 Hz, Ar-H), 6.87–6.80 (m, 2H, Ar-H), 6.59 (brs, 1H, NH), 5.06 (brs, 1H, NH), 4.45 (d, 2H, J = 6.0 Hz, CH_2), 4.10 (brs, 1H, CH), 2.68 (t, 2H, J = 7.0 Hz, CH_2), 2.19–2.15 (m, 1H, CH_2), 1.97–1.92 (m, 1H, CH_2), 1.44 (s, 9H, CH_3); ESI-MS: m/z = 405.2 $[M+H]^+$.

*4.1.7.5. Boc-hPhe-NH-benzo[d] [1,3]dioxol-5-ylmethyl (**15f**).* White solid; Yield: 97%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.31–7.28 (m, 2H, Ar-H), 7.23–7.17 (m, 3H, Ar-H), 6.77–6.73 (m, 3H, Ar-H), 6.37 (brs, 1H, NH), 5.96 (s, 2H, OCH_2O), 5.04 (brs, 1H, NH), 4.36 (brs, 2H, CH_2), 4.09 (brs, 1H, CH), 2.71 (t, 2H, J = 7.5 Hz, CH_2), 2.25–2.16 (m, 1H, CH_2), 2.00–1.91 (m, 1H, CH_2), 1.45 (s, 9H, CH_3); ESI-MS: m/z = 413.2 $[M+H]^+$.

*4.1.8. General procedure for the synthesis of deprotected C-caped amino acids **16a-f***

C-caped amino acid **15a-f** (5.0 mmol) dissolved in CH_2Cl_2 (20.0 mL) was added trifluoroacetic acid (5.0 mL) at 0 °C. The reaction mixture was then stirred for 1 h at room temperature. The volatiles were evaporated under reduced pressure and ether (30.0 mL) was added. White solid was precipitated, which was then filtrated, dried and used in the next step without further purification.

*4.1.9. General procedure for the synthesis of C-caped dipeptides **17a-f***

To a suspension of compound **13** (0.91 g, 3.0 mmol) in CH_2Cl_2 (10.0 mL), HOBt (0.41 g, 3.0 mmol) and EDCI (1.15 g, 6.0 mmol) were added at 0 °C. The reaction mixture was stirred for 30 min. Then corresponding amine hydrochloride (3.0 mmol) and diisopropylethylamine (1.1 mL, 6.0 mmol) were added. After stirring at room temperature for another 3 h, the resulting mixture was washed with aqueous $NaHCO_3$ solution (1 × 10 mL), brine (1 × 10 mL) and dried over Na_2SO_4. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography (ethyl acetate: CH_2Cl_2 = 1:5–1:1).

*4.1.9.1. Boc-Asp(NH-neopentyl)-Ala-NH-2-chlorobenzyl (**17a**).* White solid; Yield: 89%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.35–7.32 (m, 2H, Ar-H), 7.24–7.20 (m, 3H, Ar-H + NH), 7.06 (d, 1H, J = 7.5 Hz, NH), 6.15 (d, 1H, J = 6.5 Hz, NH), 6.05 (t, 1H, J = 5.5 Hz, NH), 4.55–4.47 (m, 3H, CH + CH_2), 4.41 (dd, 1H, J = 11.5, 6.5 Hz, CH_2), 2.99 (dd, 1H, J = 13.0, 6.5 Hz, CH_2), 2.92 (dd, 1H, J = 13.5, 6.5 Hz, CH_2), 2.79 (dd, 1H, J = 14.5, 4.5 Hz, CH_2), 2.64 (dd, 1H, J = 15.0, 6.5 Hz, CH_2), 1.44 (s, 9H, CH_3), 1.42 (d, 3H, J = 6.0 Hz, CH_3), 0.87 (s, 9H, CH_3); ESI-MS: m/z = 497.3 $[M+H]^+$.

*4.1.9.2. Boc-Asp(NH-neopentyl)-Leu-NH-2-chlorobenzyl (**17b**).* White solid; Yield: 80%; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.35–7.32 (m, 2H, Ar-H), 7.24–7.20 (m, 3H, Ar-H + NH), 6.90 (d, 1H, J = 8.0 Hz, NH), 6.10 (d, 1H, J = 7.0 Hz, NH), 5.93 (t, 1H, J = 6.5 Hz, NH), 4.51 (dd, 2H, J = 6.0, 2.0 Hz, CH_2), 4.47–4.41 (m, 2H, CH_2), 2.96–2.93 (m, 2H, CH_2), 2.77 (dd, 1H, J = 15.0, 5.0 Hz, CH_2), 2.66 (dd, 1H, J = 15.0, 6.0 Hz, CH_2), 1.84–1.81 (m, 1H, CH), 1.67–1.55 (m, 2H, CH_2), 1.44 (s, 9H, CH_3), 0.92 (dd, 6H, J = 22.0, 6.5 Hz, CH_2), 0.87 (s, 9H, CH_3); ESI-MS: m/z = 539.3 $[M+H]^+$.

*4.1.9.3. Boc-Asp(NH-neopentyl)-Phe-NH-2-chlorobenzyl (**17c**).* White solid; Yield: 86%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.59 (t, 1H, J = 6.0 Hz, NH), 8.16 (d, 1H, J = 8.0 Hz, NH), 7.77 (t, 1H, J = 6.5 Hz, NH), 7.43 (d, 1H, J = 7.5 Hz, NH), 7.31–7.20 (m, 7H, Ar-H), 7.12 (d, 1H, J = 7.0 Hz, Ar-H), 6.92 (d, 1H, J = 8.0 Hz, Ar-H), 4.52–4.48 (m, 1H, CH), 4.30–4.24 (m, 3H, CH + CH_2), 3.07 (dd, 1H, J = 13.5, 5.0 Hz, CH_2), 2.91 (dd, 1H, J = 14.0, 8.0 Hz, CH_2), 2.83 (dd, 1H, J = 13.0, 6.5 Hz, CH_2), 2.75 (dd, 1H, J = 13.0, 6.0 Hz, CH_2), 2.49–2.39 (m, 2H, CH_2), 1.35 (s, 9H, CH_3), 0.79 (s, 9H, CH_3); ESI-MS: m/z = 573.3 $[M+H]^+$.

*4.1.9.4. Boc-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**17d**).* White solid; Yield: 90%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.51 (t, 1H, J = 6.0 Hz, NH), 8.25 (d, 1H, J = 8.0 Hz, NH), 7.82 (t, 1H, J = 6.0 Hz, NH), 7.31–7.25 (m, 6H, Ar-H), 7.18–7.16 (m, 3H, Ar-H), 7.09 (d, 1H, J = 8.0 Hz, NH), 4.35–4.28 (m, 3H, CH_2+CH), 4.20–4.18 (m, 1H, CH), 2.82–2.71 (m, 2H, CH_2), 2.66–2.52 (m, 4H, CH_2), 2.06–1.99 (m, 1H, CH_2), 1.90–1.88 (m, 1H, CH_2), 1.37 (s, 9H, CH_3), 0.77 (s, 9H, CH_3); ESI-MS: m/z = 587.3 $[M+H]^+$.

*4.1.9.5. Boc-Asp(NH-neopentyl)-hPhe-NH-2, 4-difluorobenzyl (**17e**).* White solid; Yield: 69%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.49 (t, 1H, J = 5.5 Hz, NH), 8.18 (d, 1H, J = 8.0 Hz, NH), 7.83 (t, 1H, J = 6.0 Hz, NH), 7.78 (t, 1H, J = 6.5 Hz, NH), 7.37–7.15 (m, 6H, Ar-H), 7.06–7.00 (m, 2H, Ar-H), 4.37–4.23 (m, 3H, CH + CH_2), 4.18–4.14 (m, 1H, CH), 2.89–2.78 (m, 2H, CH_2), 2.63–2.47 (m, 4H, CH_2), 2.05–2.02 (m, 1H, CH_2), 1.89–1.82 (m, 1H, CH_2), 1.38 (s, 9H, CH_3), 0.81 (s, 9H, CH_3); ESI-MS: m/z = 589.3 $[M+H]^+$.

*4.1.9.6. Boc-Asp(NH-neopentyl)-hPhe-NH-benzo[d] [1,3]dioxol-5-ylmethyl (**17f**).* White solid; Yield: 61%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.44 (t, 1H, J = 6.0 Hz, NH), 8.13 (d, 1H, J = 8.0 Hz, NH), 7.82 (t, 1H, J = 6.0 Hz, NH), 7.26 (t, 2H, J = 7.0 Hz, Ar-H), 7.19–7.14 (m, 3H, Ar-H), 7.07 (d, 1H, J = 7.5 Hz, NH), 6.83–6.80 (m, 2H, Ar-H), 6.71 (d, 1H, J = 8.0 Hz, Ar-H), 5.97 (s, 2H, OCH_2O), 4.28 (dd, 1H, J = 15.0, 7.5 Hz, CH_2), 4.23–4.11 (m, 3H, CH + CH_2),

2.88–2.78 (m, 2H, CH_2), 2.64–2.59 (m, 2H, CH_2), 2.58–2.48 (m, 2H, CH_2), 2.06–2.00 (m, 1H, CH_2), 1.87–1.82 (m, 1H, CH_2), 1.38 (s, 9H, CH_3), 0.81 (s, 9H, CH_3); ESI-MS: m/z = 597.3 $[M+H]^+$.

*4.1.10. General procedure for the synthesis of deprotected dipeptide analogues **18a-f***

To a solution of compound **15a-f** (5.0 mmol) in CH_2Cl_2 (20.0 mL) was added trifluoroacetic acid (5.0 mL) at 0 °C. The reaction mixture was then stirred for 1 h at room temperature. The volatiles were evaporated under reduced pressure and ether (30.0 mL) was added. White solid was precipitated, which was then filtrated, dried and used in the next step without further purification.

*4.1.11. General procedure for the synthesis of target compounds **19-20***

To a suspension of corresponding piperidine-4-carboxylic acid **8a-b** (1.0 mmol) in CH_2Cl_2 (4.0 mL), HOBt (0.15 g, 1.1 mmol) and EDCI (0.29 g, 1.5 mmol) were added at 0 °C. The reaction mixture was kept at 0 °C and was stirred for 30 min. Then compound **18d** (0.12 g, 1.0 mmol) and diisopropylethylamine (0.4 mL, 2.0 mmol) were added at 0 °C. After stirring at room temperature for another 3 h, the resulting mixture was washed with aqueous $NaHCO_3$ solution (1 × 10 mL), brine (1 × 5 mL) and dried over Na_2SO_4. The solvent was evaporated in vacuo and the crude product was purified by flash chromatography (ethyl acetate: methanol = 10:1). 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.

*4.1.11.1. 1-(pyrazine-2-carbonyl)piperidine-4-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**19**).* White solid; Yield: 45%; mp: 199–201 °C; Purity: 98.5%; ^1H NMR (500 MHz, DMSO-d_6): δ = 8.81 (d, 1H, J = 3.5 Hz, pyrazine-H), 8.74 (t, 1H, J = 2.5 Hz, pyrazine-H), 8.65 (dt, 1H, J = 12.5, 1.5 Hz, pyrazine-H), 8.54 (dd, 1H, J = 8.5, 5.5 Hz, NH), 8.32 (d, 1H, J = 7.5 Hz, NH), 8.23 (d, 1H, J = 8.0 Hz, NH), 7.88 (q, 1H, J = 6.5 Hz, NH), 7.41–7.39 (m, 1H, Ar-H), 7.31–7.23 (m, 5H, Ar-H), 7.23–7.14 (m, 3H, Ar-H), 4.61 (q, 1H, J = 7.5 Hz, CH), 4.47–4.42 (m, 1H, CH_2), 4.36–4.28 (m, 2H, CH_2), 4.20–4.14 (m, 1H, CH_2), 3.69–3.64 (m, 1H, CH), 3.11–3.07 (m, 1H, CH_2), 2.92–2.87 (m, 1H, CH_2), 2.79–2.54 (m, 7H, CH + CH_2), 2.11–2.04 (m, 1H, CH_2), 1.93–1.76 (m, 2H, CH_2), 1.66–1.51 (m, 3H, CH_2), 0.76 (d, 9H, J = 6.0 Hz, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 174.22, 172.08, 171.82, 170.31, 165.10, 150.12, 145.85, 144.59, 143.75, 141.71, 136.62, 132.29, 129.42, 129.02, 128.92, 128.89, 128.75, 127.48, 126.32, 52.76, 50.46, 50.08, 46.50, 41.58, 37.67, 33.61, 32.26, 31.71, 29.30, 29.23, 28.53, 28.48, 27.61; ESI-MS: m/z = 704.1 $[M+H]^+$.

*4.1.11.2. 1-(4-fluorobenzoyl)piperidine-4-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**20**).* White solid; Yield: 42%; mp: 187–189 °C; Purity: 99.1%; ^1H NMR (500 MHz, DMSO-d_6): δ = 8.55 (t, 1H, J = 6.0 Hz, NH), 8.32 (d, 1H, J = 8.0 Hz, NH), 8.23 (d, 1H, J = 8.0 Hz, NH), 7.89 (t, 1H, J = 6.0 Hz, NH), 7.44–7.40 (m, 3H, Ar-H), 7.32–7.24 (m, 7H, Ar-H), 7.19–7.13 (m, 3H, Ar-H), 4.61 (q, 1H, J = 8.0 Hz, CH), 4.48–4.28 (m, 3H, CH_2), 4.19–4.16 (m, 1H, CH_2), 3.64–3.56 (m, 1H, CH), 3.10–2.99 (m, 1H, CH_2), 2.85–2.55 (m, 7H, CH + CH_2), 2.11–2.03 (m, 1H, CH_2), 1.92–1.81 (m, 1H, CH_2), 1.79–1.43 (m, 5H, CH_2), 0.76 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 174.27, 172.07, 171.81, 170.30, 168.56, 163.87, 161.91, 141.73, 136.61, 133.09, 132.29, 129.74, 129.67, 129.42, 129.01, 128.90, 128.74, 127.48, 126.31, 115.93, 115.76, 52.77, 50.44, 50.07, 41.92, 41.76, 37.66, 33.63, 32.26, 31.72, 30.63, 27.61; ESI-MS: m/z = 720.1 $[M+H]^+$.

*4.1.12. General procedure for the synthesis of target compounds **21-43***

Corresponding dipeptide fragment **18a-f** (3.0 mmol) was dissolved in CH_2Cl_2 (5.0 mL) and saturated aqueous $NaHCO_3$ (5.0 mL) and was cooled to 0 °C. Triphosgene (0.30 g, 1.0 mmol) was added in one portion and the mixture was stirred at 0 °C for 10 min. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 10 mL). Then the organic phase was combined, dried over Na_2SO_4, evaporated in vacuo and used directly. To a solution of corresponding amine TFA salt (**7a-b**, **9a-b** and **10a-i**, 2.0 mmol) was added diisopropylethylamine (0.8 mL, 4.0 mmol). Then the former obtained isocyanate was dissolved in CH_2Cl_2 (3.0 mL) and added into the reaction mixture. After 3 h'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=50:1–10:1). Purity test condition was the same with that of the compounds **19-20**.

*4.1.12.1. 4-(pyrazine-2-carbonyl)piperazine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**21**).* White solid; Yield: 79%; mp: 205–207 °C; Purity: 98.8%; ^1H NMR (500 MHz, DMSO-d_6): δ = 8.86 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.77 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.69 (dd, 1H, J = 2.5, 1.5 Hz, pyrazing-H), 8.52 (t, 1H, J = 6.0 Hz, NH), 8.27 (d, 1H, J = 7.5 Hz, NH), 7.82 (t, 1H, J = 6.0 Hz, NH), 7.43–7.41 (m, 1H, Ar-H), 7.33–7.25 (m, 5H, Ar-H), 7.20–7.15 (m, 3H, Ar-H), 6.89 (d, 1H, J = 8.0 Hz, NH), 4.47 (q, 1H, J = 7.5 Hz, CH), 4.32 (d, 2H, J = 6.0 Hz, CH_2), 4.20–4.16 (m, 1H, CH), 3.65–3.60 (m, 2H, CH_2), 3.47–3.32 (m, 6H, CH_2), 2.85–2.76 (m, 2H, CH_2), 2.70–2.53 (m, 4H, CH_2), 2.10–2.02 (m, 1H, CH_2), 1.94–1.88 (m, 1H, CH_2), 0.79 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 172.73, 172.14, 170.59, 165.37, 157.46, 149.56, 146.05, 145.02, 143.68, 141.84, 136.67, 132.35, 129.44, 129.08, 128.94, 128.87, 128.74, 127.49, 126.28, 52.88, 52.44, 50.16, 46.77, 44.24, 43.67, 42.07, 37.89, 33.72, 32.28, 31.77, 27.63; ESI-MS: m/z = 705.1 $[M+H]^+$.

*4.1.12.2. 4-(4-fluorobenzoyl)piperazine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**22**).* White solid; Yield: 87%; mp: 174–176 °C; Purity: 98.9%; ^1H NMR (500 MHz, DMSO-d_6): δ = 8.52 (t, 1H, J = 6.0 Hz, NH), 8.27 (d, 1H, J = 7.5 Hz, NH), 7.82 (t, 1H, J = 6.0 Hz, NH), 7.49–7.47 (m, 2H, Ar-H), 7.43–7.41 (m, 1H, Ar-H), 7.31–7.25 (m, 7H, Ar-H), 7.19–7.16 (m, 3H, Ar-H), 6.88 (d, 1H, J = 7.5 Hz, NH), 4.47 (q, 1H, J = 7.5 Hz, CH), 4.33 (d, 2H, J = 6.0 Hz, CH_2), 4.21–4.16 (m, 1H, CH), 3.68–3.20 (m, 8H, piperazine-H), 2.85–2.75 (m, 2H, CH_2), 2.68–2.54 (m, 4H, CH_2), 2.10–2.03 (m, 1H, CH_2), 1.94–1.86 (m, 1H, CH_2), 0.79 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 172.74, 172.14, 170.59, 168.82, 164.29, 161.83, 157.47, 141.84, 136.67, 132.59, 132.56, 132.35, 130.13, 130.04, 129.43, 129.06, 128.94, 128.87, 128.75, 127.49, 126.28, 115.97, 115.76, 52.89, 52.41, 50.16, 43.90, 37.89, 33.73, 32.27, 31.78, 27.63; ESI-MS: m/z = 721.1 $[M+H]^+$.

*4.1.12.3. 4-(Pyrazine-2-carboxamido)piperidine-1-carbonyl-Asp(NH-neopentyl)-Ala-NH-2-chlorobenzyl (**23**).* White solid; Yield: 17%; mp: 171–173 °C; Purity: 99.3%; ^1H NMR (500 MHz, DMSO-d_6): δ = 9.17 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.86 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.83 (d, 1H, J = 8.5 Hz, NH), 8.69 (dd, 1H, J = 2.0, 1.5 Hz, pyrazine-H), 8.53 (t, 1H, J = 6.0 Hz, NH), 8.18 (d, 1H, J = 7.5 Hz, NH), 7.79 (t, 1H, J = 6.0 Hz, NH), 7.42–7.40 (m, 1H, Ar-H), 7.32–7.26 (m, 3H, Ar-H), 6.69 (d, 1H, J = 7.5 Hz, NH), 4.39–4.25 (m, 4H, CH + CH_2), 4.01–3.92 (m, 3H, CH_2+CH), 2.85–2.72 (m, 4H, CH_2), 2.61–2.57 (dd, 1H, J = 14.5, 6.5 Hz, CH_2), 2.54–2.50 (dd, 1H, J = 14.5, 8.0 Hz, CH_2), 1.73–1.71 (m, 2H, CH_2), 1.59–1.46 (m, 2H, CH_2); 1.28 (d, 3H, J = 7.0 Hz, CH_3); 0.78 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.89, 172.47, 170.57, 162.65, 157.28, 147.88, 145.42, 144.08,

143.68, 136.64, 132.33, 129.44, 129.01, 128.45, 127.54, 52.48, 50.17, 49.02, 47.10, 43.24. 43.12, 38.17, 32.27, 31.46, 27.63, 18.38; ESI-MS: m/z = 629.1 $[M+H]^+$.

*4.1.12.4. 4-(Pyrazin-2-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-Ala-NH-2-chlorobenzyl (**24**).* White solid; Yield: 43%; mp: 186–188 °C; Purity: 98.2%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.79 (s, 1H, NH), 9.33 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.51 (t, 1H, J = 6.0 Hz, NH), 8.40 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.35 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.22 (d, 1H, J = 7.5 Hz, NH), 7.78 (t, 1H, J = 6.0 Hz, NH), 7.42–7.41 (m, 1H, Ar-H), 7.33–7.25 (m, 3H, Ar-H), 6.65–6.63 (d, 1H, J = 7.5 Hz, NH), 4.38 (q, 1H, J = 8.0 Hz, CH), 4.31 (d, 2H, J = 6.0 Hz, CH_2), 4.27 (t, 1H, J = 7.5 Hz, CH), 4.01–3.95 (m, 2H, CH_2), 2.84–2.52 (m, 7H, CH_2+CH), 1.80–1.76 (m, 2H, CH_2), 1.56–1.42 (m, 2H, CH_2), 1.29 (d, 3H, J = 7.0 Hz, CH_3), 0.78 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 174.67, 172.48, 170.58, 157.31, 149.35, 143.03, 140.07, 136.75, 136.68, 132.31, 129.42, 128.97, 128.91, 127.52, 52.38, 50.15, 49.06, 43.53, 43.41, 42.48, 38.22, 32.27, 28.40, 27.62, 18.52; ESI-MS: m/z = 629.1 $[M+H]^+$.

*4.1.12.5. 4-(Pyrazine-2-carboxamido)piperidine-1-carbonyl-Asp(NH-neopentyl)-Leu-NH-2-chlorobenzyl (**25**).* White solid; Yield: 26%; mp: 139–141 °C; Purity: 98.9%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.18 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.86 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.82 (d, 1H, J = 8.5 Hz, NH), 8.70 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.52 (t, 1H, J = 6.0 Hz, NH), 8.05 (d, 1H, J = 8.0 Hz, NH), 7.79 (t, 1H, J = 6.0 Hz, NH), 7.42–7.40 (m, 1H, Ar-H), 7.31–7.25 (m, 3H, Ar-H), 6.74 (d, 1H, J = 7.5 Hz, NH), 4.39–4.25 (m, 4H, CH_2+CH), 4.02–3.91 (m, 3H, CH_2+CH), 2.86–2.71 (m, 4H, CH_2), 2.61–2.52 (m, 2H, CH_2), 1.73–1.71 (m, 2H, CH_2), 1.59–1.44 (m, 5H, CH_2+CH), 0.90 (d, 3H, J = 7.0 Hz, CH_3), 0.84 (d, 3H, J = 6.5 Hz, CH_3), 0.79 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.78, 172.74, 170.52, 162.62, 157.39, 147.92, 145.38, 144.09, 143.70, 136.65, 132.30, 129.43, 128.95, 127.53, 52.53, 51.74, 50.13, 47.08, 43.24, 43.08, 40.75, 37.85, 32.31, 31.45, 27.62, 24.66, 23.65, 21.84; ESI-MS: m/z = 671.1 $[M+H]^+$.

*4.1.12.6. 4-(Pyrazin-2-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-Leu-NH-2-chlorobenzyl (**26**).* White solid; Yield: 27%; mp: 158–160 °C; Purity: 99.5%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.80 (s, 1H, NH), 9.33 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.52 (t, 1H, J = 5.5 Hz, NH), 8.40 (d, 1H, J = 1.5 Hz, pyrazing-H), 8.35 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.07 (d, 1H, J = 8.0 Hz, NH), 7.78 (t, 1H, J = 6.0 Hz, NH), 7.43–7.40 (m, 1H, Ar-H), 7.32–7.26 (m, 3H, Ar-H), 6.71 (d, 1H, J = 7.0 Hz, NH), 4.40 (q, 1H, J = 8.0 Hz, CH), 4.36–4.26 (m, 3H, CH + CH_2), 4.00–3.95 (m, 2H, CH_2), 2.86–2.75 (m, 2H, CH_2), 2.74–2.50 (m, 5H, CH + CH_2), 1.80–1.73 (m, 2H, CH_2), 1.66–1.43 (m, 5H, CH + CH_2), 0.90 (d, 3H, J = 6.5 Hz, CH_3), 0.84 (d, 3H, J = 6.5 Hz, CH_3), 0.80 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 174.65, 172.71, 172.70, 170.60, 157.41, 149.36, 143.04, 140.06, 136.73, 136.69, 132.32, 129.41, 129.01, 128.90, 127.51, 52.40, 51.79, 50.16, 43.52, 43.43, 42.48, 37.83, 32.29, 28.40, 28.35, 27.64, 24.68, 23.61, 21.80; ESI-MS: m/z = 671.2 $[M+H]^+$.

*4.1.12.7. 4-(Pyrazine-2-carboxamido)piperidine-1-carbonyl-Asp(NH-neopentyl)-Phe-NH-2-chlorobenzyl (**27**).* White solid; Yield: 25%; mp: 156–158 °C; Purity: 99.2%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.18 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.86 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.81 (d, 1H, J = 8.5 Hz, NH), 8.70 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.57 (t, 1H, J = 6.0 Hz, NH), 8.12 (d, 1H, J = 8.0 Hz, NH), 7.75 (t, 1H, J = 6.0 Hz, NH), 7.42–7.40 (m, 1H, Ar-H), 7.25–7.19 (m, 7H, Ar-H), 7.16–7.14 (m, 1H, Ar-H), 6.70 (d, 1H, J = 7.5 Hz, NH), 4.46 (td, 1H, J = 8.5, 5.0 Hz, CH), 4.37–4.32 (m, 3H, CH_2 +CH), 4.02–3.88 (m, 3H, CH_2 +CH), 3.09 (dd, 1H, J = 14.0, 5.0 Hz, CH_2), 2.94 (dd, 1H, J = 13.5, 9.0 Hz, CH_2), 2.86 (q, 1H, J = 6.5 Hz, CH_2), 2.80–2.69 (m, 3H, CH_2), 2.52–2.44 (m, 2H, CH_2), 1.72–1.70 (m, 2H, CH_2); 1.49 (qd, 2H, J = 11.5, 3.0 Hz, CH_2), 0.79 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.58, 171.46, 170.53, 162.66, 157.38, 147.89, 145.42, 144.08, 143.68, 138.30, 136.46, 132.31, 129.64, 129.40, 129.09, 128.94, 128.59, 127.52, 126.75, 54.70, 52.47. 50.17, 47.07, 43.23, 43.09, 37.78, 37.49, 32.31, 31.41, 27.64; ESI-MS: m/z = 705.1 $[M+H]^+$.

*4.1.12.8. 4-(Pyrazin-2-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-Phe-NH-2-chlorobenzyl (**28**).* White solid; Yield: 45%; mp: 165–167 °C; Purity: 98.9%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.80 (s, 1H, NH), 9.34 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.57 (t, 1H, J = 6.0 Hz, NH), 8.40 (dd, 1H, J = 2.5, 1.5 Hz, pyrazing-H), 8.35 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.16 (d, 1H, J = 8.0 Hz, NH), 7.76 (t, 1H, J = 6.0 Hz, NH), 7.43–7.41 (m, 1H, Ar-H), 7.30–7.18 (m, 7H, Ar-H), 7.17–7.15 (m, 1H, Ar-H), 6.66 (d, 1H, J = 7.5 Hz, NH), 4.49–4.44 (m, 1H, CH), 4.38 (q, 1H, J = 8.0 Hz, CH_2), 4.32 (d, 2H, J = 5.5 Hz, CH_2), 4.00–3.91 (m, 2H, CH + CH_2), 3.10 (dd, 1H, J = 14.0, 5.0 Hz, CH_2), 2.93 (dd, 1H, J = 13.5, 8.0 Hz, CH_2), 2.85 (dd, 1H, J = 13.0, 6.5 Hz, CH_2), 2.77 (dd, 1H, J = 14.0, 5.5 Hz, CH_2), 2.73–2.50 (m, 3H, CH + CH_2), 2.52–2.43 (m, 2H, CH_2), 1.80–1.75 (m, 2H, CH_2), 1.49–1.41 (m, 2H, CH_2), 0.80 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 174.71, 172.62, 171.53, 170.60, 157.38, 149.30, 143.08, 140.08, 138.25, 136.71, 136.40, 132.30, 129.60, 129.39, 129.05, 128.95, 128.62, 127.52, 126.79, 54.76, 52.40, 50.17, 43.45, 43.35, 42.43, 37.80, 37.38, 32.29, 28.35, 28.30, 27.60; ESI-MS: m/z = 705.1 $[M+H]^+$.

*4.1.12.9. 4-(Pyrazine-2-carboxamido)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**29**).* White solid; Yield: 17%; mp: 183–185 °C; Purity: 97.9%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.17 (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), 8.70 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.53 (t, 1H, J = 6.0 Hz, NH), 8.22 (d, 1H, J = 8.0 Hz, NH), 7.81 (t, 1H, J = 6.0 Hz, NH), 7.42–7.40 (m, 1H, Ar-H), 7.28–7.25 (m, 5H, Ar-H), 7.18–7.17 (m, 3H, Ar-H), 6.78 (d, 1H, J = 7.5 Hz, NH), 4.42 (q, 1H, J = 7.5 Hz, CH), 4.33 (d, 2H, J = 6.0 Hz, CH_2), 4.20–4.16 (m, 1H, CH), 4.00–3.95 (m, 3H, CH_2+CH), 2.85–2.74 (m, 4H, CH_2), 2.67–2.53 (m, 4H, CH_2), 2.09–2.02 (m, 1H, CH_2), 1.93–1.86 (m, 1H, CH_2), 1.74–1.72 (m, 2H, CH_2), 1.59–1.45 (m, 2H, CH_2), 0.78 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.91, 172.15, 170.64, 162.65, 157.44, 147.89, 145.41, 144.06, 143.68, 141.86, 136.65, 132.36, 129.44, 129.11, 128.96, 128.88, 128.74, 127.50, 126.26, 52.85, 52.59, 50.19, 47.11, 43.26, 43.14, 37.91, 33.80, 32.27, 31.75, 31.47, 27.63; ESI-MS: m/z = 719.1 $[M+H]^+$.

*4.1.12.10. 4-(Pyrazin-2-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**30**).* White solid; Yield: 15%; mp: 191–193 °C; Purity: 98.4%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.77 (s, 1H, NH), 9.33 (d, 1H, J = 3.5 Hz, pyrazine-H), 8.52 (t, 1H, J = 6.0 Hz, NH), 8.39 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.34 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.24 (d, 1H, J = 7.5 Hz, NH), 7.81 (t, 1H, J = 6.0 Hz, NH), 7.42–7.40 (m, 1H, Ar-H), 7.32–7.25 (m, 5H, Ar-H), 7.18–7.15 (m, 3H, Ar-H), 6.75 (d, 1H, J = 7.5 Hz, NH), 4.44 (q, 1H, J = 7.5 Hz, CH), 4.32 (d, 2H, J = 5.5 Hz, CH_2), 4.19–4.15 (m, 1H, CH), 4.05–3.98 (m, 2H, CH_2), 2.85–2.54 (m, 9H, CH_2+CH), 2.10–2.03 (m, 1H, CH_2), 1.93–1.87 (m, 1H, CH_2), 1.79–1.75 (m, 2H, CH_2); 1.53–1.45 (m, 2H, CH_2), 0.78 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 174.65, 172.89, 172.15, 170.66, 157.45, 149.35, 143.03, 141.84, 140.06, 136.75, 136.67, 132.33, 129.43, 129.08, 128.93, 128.89, 128.73, 127.50, 126.25, 52.83, 52.46, 50.16, 43.55, 43.46, 42.47, 37.91, 33.71, 32.27, 31.75, 28.42, 28.37, 27.64; ESI-MS: m/z = 719.1 $[M+H]^+$.

*4.1.12.11. 4-(Pyrazine-2-carboxamido)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2, 4-difluorobenzyl (**31**).* White solid; Yield: 50%; mp: 153–155 °C; Purity: 99.3%; ^{1}H NMR (500 MHz,

DMSO-d_6): δ = 9.18 (d, 1H, J = 1.0 Hz, pyrazine-H), 8.86 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.77 (d, 1H, J = 8.0 Hz, NH), 8.71 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.51 (t, 1H, J = 5.5 Hz, NH), 8.14 (d, 1H, J = 8.0 Hz, NH), 7.82 (t, 1H, J = 6.0 Hz, NH), 7.35 (q, 1H, J = 9.0 Hz, Ar-H), 7.28–7.25 (m, 2H, Ar-H), 7.21–7.16 (m, 4H, Ar-H), 7.02 (dt, 1H, J = 8.5, 2.0 Hz, Ar-H), 6.76 (d, 1H, J = 7.5 Hz, NH), 4.40 (q, 1H, J = 7.0 Hz, CH), 4.30–4.27 (m, 2H, CH_2), 4.18–4.13 (m, 1H, CH), 4.03–3.95 (m, 3H, CH + CH_2), 2.87–2.78 (m, 4H, CH_2), 2.65–2.55 (m, 4H, CH_2), 2.08–2.00 (m, 1H, CH_2), 1.89–1.84 (m, 1H, CH_2), 1.76–1.73 (m, 2H, CH_2), 1.59–1.48 (m, 2H, CH_2), 0.81 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.83, 172.04, 170.63, 162.65, 157.44, 147.89, 145.41, 144.07, 143.68, 141.85, 131.06, 128.86, 128.73, 126.26, 122.83, 111.70, 104.22, 103.96, 103.71, 52.75, 52.60, 50.18, 47.11, 43.26, 43.14, 37.89, 36.00, 35.95, 33.80, 32.29, 31.71, 31.48, 27.64; ESI-MS: m/z = 721.4 $[M+H]^+$.

*4.1.12.12. 4-(Pyrazin-2-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2, 4-difluorobenzyl (**32**).* White solid; Yield: 38%; mp: 168–170 °C; Purity: 99.2%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.76 (s, 1H, NH), 9.33 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.49 (t, 1H, J = 6.0 Hz, NH), 8.40 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.35 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.17 (d, 1H, J = 7.5 Hz, NH), 7.81 (t, 1H, J = 6.0 Hz, NH), 7.35 (q, 1H, J = 9.0 Hz, Ar-H), 7.28–7.13 (m, 6H, Ar-H), 7.02 (dt, 1H, J = 8.5, 2.0 Hz, Ar-H), 6.74 (d, 1H, J = 7.5 Hz, NH), 4.42 (q, 1H, J = 7.0 Hz, CH), 4.30–4.27 (m, 2H, CH_2), 4.17–4.13 (m, 1H, CH), 4.05–3.98 (m, 2H, CH_2), 2.85–2.54 (m, 9H, CH + CH_2), 2.06–2.03 (m, 1H, CH_2), 1.89–1.77 (m, 3H, CH_2), 1.53–1.46 (m, 2H, CH_2), 0.81 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 174.65, 172.83, 172.06, 170.65, 157.46, 149.36, 143.02, 141.84, 140.06, 136.75, 131.04, 128.87, 128.72, 126.24, 122.85, 111.69, 104.20, 103.95, 103.69, 52.74, 52.50, 50.17, 43.55, 43.46, 42.48, 37.90, 35.98, 35.95, 33.71, 32.29, 31.73, 28.42, 28.38, 27.64; ESI-MS: m/z = 721.4 $[M+H]^+$.

*4.1.12.13. 4-(Pyrazine-2-carboxamido)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-benzo[d][1,3]dioxol-5-ylmethyl (**33**).* White solid; Yield: 39%; mp: 174–176 °C; Purity: 99.4%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.17 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.86 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.54 (d, 1H, J = 8.5 Hz, NH), 8.69 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.44 (t, 1H, J = 6.0 Hz, NH), 8.09 (d, 1H, J = 8.0 Hz, NH), 7.80 (t, 1H, J = 6.0 Hz, NH), 7.27–7.14 (m, 5H, Ar-H), 6.82–6.76 (m, 3H, Ar-H), 6.70 (d, 1H, J = 8.0 Hz, NH), 5.95 (s, 2H, OCH_2O), 4.39 (q, 1H, J = 7.0 Hz, CH), 4.24–4.14 (m, 3H, CH + CH_2), 4.01–3.95 (m, 3H, CH_2+CH), 2.89–2.76 (m, 4H, CH_2), 2.65–2.52 (m, 4H, CH_2), 2.07–1.99 (m, 1H, CH_2), 1.88–1.81 (m, 1H, CH_2), 1.75–1.73 (m, 2H, CH_2), 1.59–1.48 (m, 2H, CH_2), 0.81 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 172.74, 171.70, 170.62, 162.65, 157.43, 147.89, 147.66, 146.44, 145.41, 144.07, 143.68, 141.90, 133.77, 128.85, 128.73, 126.25, 120.78, 108.36, 108.28, 101.22, 52.72, 52.66, 50.22, 47.11, 43.28, 43.16, 42.35, 37.91, 33.94, 32.31, 31.72, 31.49, 27.66; ESI-MS: m/z = 729.3 $[M+H]^+$.

*4.1.12.14. 4-(Pyrazin-2-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH- benzo[d][1,3]dioxol-5-ylmethyl (**34**).* White solid; Yield: 13%; mp: 183–185 °C; Purity: 98.9%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.76 (s, 1H, NH), 9.33 (d, 1H, J = 1.5 Hz, pyrazine-H), 8.43 (t, 1H, J = 6.0 Hz, NH), 8.40 (dd, 1H, J = 2.5, 1.5 Hz, pyrazine-H), 8.35 (d, 1H, J = 2.5 Hz, pyrazine-H), 8.12 (d, 1H, J = 7.5 Hz, NH), 7.81 (t, 1H, J = 6.0 Hz, NH), 7.28–7.24 (m, 2H, Ar-H), 7.18–7.15 (m, 3H, Ar-H), 6.83–6.81 (m, 2H, Ar-H), 6.75–6.71 (m, 2H, NH + Ar-H), 5.96 (s, 2H, OCH_2O), 4.42 (q, 1H, J = 7.0 Hz, CH), 4.23–4.12 (m, 3H, CH + CH_2), 4.05–3.99 (m, 2H, CH_2), 2.90–2.56 (m, 9H, CH + CH_2), 2.08–2.01 (m, 1H, CH_2), 1.89–1.72 (m, 3H, CH_2), 1.52–1.43 (m, 2H, CH_2), 0.82 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ = 174.65, 172.76, 171.71, 170.64, 157.46, 149.36, 147.67, 146.43, 143.03, 141.89, 140.06, 136.75, 133.80, 128.87, 128.71, 126.23, 120.76, 108.35, 108.27, 101.21, 52.72, 52.55, 50.20, 43.55, 43.46, 42.48, 42.35, 37.92, 33.83, 32.31, 31.75, 28.42, 27.67; ESI-MS: m/z = 729.4 $[M+H]^+$.

*4.1.12.15. 4-(4-fluorobenzamido) piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**35**).* White solid; Yield: 28%; mp: 159–161 °C; Purity: 99.3%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.52 (t, 1H, J = 6.0 Hz, NH), 8.29 (d, 1H, J = 8.0 Hz, NH), 8.23 (d, 1H, J = 8.0 Hz, NH), 7.93–7.89 (m, 2H, Ar-H), 7.80 (t, 1H, J = 6.0 Hz, NH), 7.43–7.41 (m, 1H, Ar-H), 7.34–7.26 (m, 7H, Ar-H), 7.20–7.16 (m, 3H, Ar-H), 6.75 (d, 1H, J = 7.5 Hz, NH), 4.43 (q, 1H, J = 7.5 Hz, CH), 4.33 (d, 2H, J = 6.0 Hz, CH_2), 4.22–4.17 (m, 1H, CH), 4.00–3.93 (m, 3H, CH + CH_2), 2.87–2.77 (m, 4H, CH_2), 2.68–2.55 (m, 4H, CH_2), 2.10–2.05 (m, 1H, CH_2), 1.95–1.88 (m, 1H, CH_2), 1.78–1.73 (m, 2H, CH_2), 1.48–1.35 (m, 2H, CH_2), 0.80 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 174.65, 172.97, 172.17, 170.62, 169.25, 165.02, 157.48, 141.85, 136.66, 132.34, 132.15, 131.57, 130.44, 129.44, 129.07, 128.90, 128.75, 127.51, 126.28, 115.64, 115.42, 53.79, 53.32, 52.86, 52.53, 50.17, 47.30, 43.29, 43.10, 37.95, 36.15, 33.74, 32.28, 31.76, 27.63; ESI-MS: m/z = 735.3 $[M+H]^+$.

*4.1.12.16. 4-(4-fluorophenylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**36**).* White solid; Yield: 35%; mp: 161–163 °C; Purity: 98.2%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.94 (s, 1H, NH), 8.52 (t, 1H, J = 6.0 Hz, NH), 8.23 (d, 1H, J = 8.0 Hz, NH), 7.80 (t, 1H, J = 6.0 Hz, NH), 7.63–7.60 (m, 2H, Ar-H), 7.43–7.41 (m, 1H, Ar-H), 7.33–7.26 (m, 5H, Ar-H), 7.19–7.11 (m, 5H, Ar-H), 6.74 (d, 1H, J = 7.5 Hz, NH), 4.45 (q, 1H, J = 7.5 Hz, CH), 4.33 (d, 2H, J = 6.0 Hz, CH_2), 4.21–4.17 (m, 1H, CH), 4.06–3.99 (m, 2H, CH_2), 2.87–2.56 (m, 9H, CH + CH_2), 2.08–2.06 (m, 1H, CH_2), 1.93–1.88 (m, 1H, CH_2), 1.77–1.73 (m, 2H, CH_2), 1.57–1.45 (m, 2H, CH_2), 0.80 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 173.40, 172.92, 172.17, 170.67, 157.47, 141.84, 136.68, 136.17, 132.34, 129.43, 129.08, 128.90, 128.73, 127.49, 126.26, 121.35, 121.28, 115.74, 115.52, 52.85, 52.48, 50.17, 43.60, 43.50, 43.11, 37.93, 33.72, 32.27, 31.77, 28.59, 27.64; ESI-MS: m/z = 735.1 $[M+H]^+$.

*4.1.12.17. 4-(4-methoxyphenylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**37**).* White solid; Yield: 16%; mp: 157–159 °C; Purity: 98.8%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 9.74 (s, 1H, NH), 7.76 (t, 1H, J = 6.0 Hz, NH), 7.52 (d, 2H, J = 8.0 Hz, Ar-H), 7.45–7.43 (m, 1H, Ar-H), 7.35–7.16 (m, 6H, Ar-H), 6.86 (d, 2H, J = 9.0 Hz, Ar-H), 6.81 (d, 1H, J = 7.5 Hz, NH), 4.46–4.36 (m, 2H, CH + CH), 4.01–3.95 (m, 2H, CH_2), 3.72 (s, 3H, CH_3), 3.40–3.32 (m, 2H, CH_2), 2.95 (dd, 1H, J = 13.0, 6.5 Hz, CH_2), 2.82 (dd, 1H, J = 13.0, 6.5 Hz, CH_2), 2.74–2.56 (m, 6H, CH_2), 2.48–2.44 (m, 1H, CH), 1.94–1.88 (m, 1H, CH_2), 1.75–1.69 (m, 2H, CH_2), 1.52–1.46 (m, 2H, CH_2), 0.84 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 175.36, 173.51, 173.01, 169.84, 157.22, 155.54, 142.38, 136.90, 132.95, 132.52, 129.56, 129.29, 129.06, 128.77, 128.74, 127.59, 126.17, 121.14, 114.24, 55.62, 54.80, 52.17, 51.52, 50.20, 43.61, 43.54, 43.11, 37.87, 37.27, 32.41, 31.92, 28.63, 27.66, 21.61; ESI-MS: m/z = 747.1 $[M+H]^+$.

*4.1.12.18. 4-(4-(trifluoromethyl)phenylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**38**).* White solid; Yield: 14%; mp: 138–140 °C; Purity: 98.6%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.02 (s, 1H, NH), 8.51 (t, 1H, J = 6.0 Hz, NH), 8.21 (d, 1H, J = 8.0 Hz, NH), 7.80 (t, 1H, J = 6.0 Hz, NH), 7.56 (d, 2H, J = 8.0 Hz, Ar-H), 7.43–7.41 (m, 1H, Ar-H), 7.33–7.28 (m, 6H, Ar-H), 7.19–7.15 (m, 3H, Ar-H), 6.73 (d, 1H, J = 7.5 Hz, NH), 4.44 (q, 1H, J = 7.0 Hz, CH), 4.33 (d, 2H, J = 6.0 Hz, CH_2), 4.21–4.16 (m, 1H, CH), 4.06–3.99 (m, 2H, CH_2), 2.84–2.57 (m, 9H, CH + CH_2), 2.10–2.03 (m, 1H, CH_2), 1.94–1.88 (m, 1H, CH_2), 1.76–1.73 (m, 2H, CH_2), 1.52–1.44 (m, 2H, CH_2), 0.79 (s, 9H, CH_3); ^{13}C NMR (100 MHz,

DMSO): δ = 173.82, 172.90, 172.16, 170.66, 157.45, 141.84, 136.67, 132.34, 130.20, 129.43, 129.08, 128.93, 128.89, 128.73, 127.49, 126.25, 126.13, 126.09, 118.92, 52.84, 52.46, 50.17, 43.57, 43.47, 43.19, 37.92, 33.72, 32.27, 31.75, 28.53, 27.63; ESI-MS: *m*/*z* = 785.1 $[M+H]^+$.

*4.1.12.19. 4-(pyridin-3-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**39**).* White solid; Yield: 31%; mp: 163–165 °C; Purity: 99.2%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.08 (s, 1H, NH), 8.71 (d, 1H, *J* = 2.5 Hz, pyridine-H), 8.49 (t, 1H, *J* = 6.0 Hz, NH), 8.23–8.20 (m, 2H, pyridine-H + NH), 8.02 (dt, 1H, *J* = 10.5, 2.0 Hz, pyridine-H), 7.78 (t, 1H, *J* = 6.0 Hz, NH), 7.41–7.39 (m, 1H, Ar-H), 7.32–7.23 (m, 6H, Ar-H), 7.17–7.13 (m, 3H, pyridine-H + Ar-H), 6.72 (d, 1H, *J* = 7.5 Hz, NH), 4.43 (q, 1H, *J* = 6.5 Hz, CH), 4.31 (d, 2H, *J* = 5.5 Hz, CH_2), 4.19–4.14 (m, 1H, CH), 4.04–3.97 (m, 2H, CH_2), 2.82–2.52 (m, 9H, CH + CH_2), 2.08–2.01 (m, 1H, CH_2), 1.92–1.87 (m, 1H, CH_2), 1.77–1.73 (m, 2H, CH_2), 1.54–1.44 (m, 2H, CH_2), 0.77 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 174.09, 172.91, 172.16, 170.69, 157.47, 144.51, 141.84, 141.26, 136.67, 136.37, 132.34, 129.43, 129.08, 128.94, 128.89, 128.73, 127.50, 126.51, 126.26, 124.03, 52.84, 52.47, 50.17, 43.56, 43.47, 43.07, 37.93, 33.72, 32.28, 31.76, 28.54, 28.51, 27.64; ESI-MS: *m*/*z* = 718.2 $[M+H]^+$.

*4.1.12.20. 4-(pyridin-2-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**40**).* White solid; Yield: 26%; mp: 140–142 °C; Purity: 99.1%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.43 (s, 1H, NH), 8.51 (t, 1H, *J* = 6.0 Hz, NH), 8.32–8.30 (m, 1H, pyridine-H), 8.22 (d, 1H, *J* = 7.5 Hz, NH), 8.07 (d, 1H, *J* = 8.0 Hz, pyridine-H), 7.81–7.74 (m, 2H, NH + pyridine-H), 7.43–7.41 (m, 1H, Ar-H), 7.33–7.17 (m, 7H, Ar-H), 7.10–7.07 (m, 1H, pyridine-H), 6.73 (d, 1H, *J* = 7.5 Hz, NH), 4.44 (q, 1H, *J* = 7.0 Hz, CH), 4.32 (d, 2H, *J* = 6.0 Hz, CH_2), 4.21–4.16 (m, 1H, CH), 4.05–3.98 (m, 2H, CH_2), 2.87–2.55 (m, 9H, CH + CH_2), 2.10–2.03 (m, 1H, CH_2), 1.95–1.87 (m, 1H, CH_2), 1.77–1.74 (m, 2H, CH_2), 1.53–1.44 (m, 2H, CH_2), 0.80 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 174.45, 172.92, 172.17, 170.67, 157.45, 152.61, 148.36, 141.84, 138.52, 136.67, 132.33, 129.43, 129.08, 128.93, 128.90, 128.73, 127.50, 126.26, 119.72, 113.97, 52.84, 52.49, 52.17, 50.17, 43.61, 43.50, 42.67, 37.92, 33.70, 32.28, 31.76, 28.53, 28.48, 27.64; ESI-MS: *m*/*z* = 718.1 $[M+H]^+$.

*4.1.12.21. 4-(isoxazol-3-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**41**).* White solid; Yield: 38%; mp: 149–151 °C; Purity: 98.9%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 11.00 (s, 1H, NH), 8.77 (d, 1H, *J* = 2.0 Hz, isoxazol-H), 8.51 (t, 1H, *J* = 5.5 Hz, NH), 8.22 (d, 1H, *J* = 7.5 Hz, NH), 7.79 (t, 1H, *J* = 6.0 Hz, NH), 7.43–7.41 (m, 1H, Ar-H), 7.34–7.25 (m, 5H, Ar-H), 7.19–7.15 (m, 3H, Ar-H), 6.92 (d, 1H, *J* = 1.5 Hz, isoxazol-H), 6.73 (d, 1H, *J* = 7.5 Hz, NH), 4.44 (q, 1H, *J* = 7.0 Hz, CH), 4.33 (d, 2H, *J* = 6.0 Hz, CH_2), 4.21–4.16 (m, 1H, CH), 4.04–3.97 (m, 2H, CH_2), 2.84–2.55 (m, 9H, CH + CH_2), 2.10–2.04 (m, 1H, CH_2), 1.94–1.88 (m, 1H, CH_2), 1.77–1.73 (m, 2H, CH_2), 1.52–1.41 (m, 2H, CH_2), 0.80 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 173.84, 172.89, 172.16, 170.66, 160.43, 158.09, 157.44, 141.84, 136.67, 132.34, 129.43, 129.09, 128.94, 128.89, 128.73, 127.49, 126.26, 99.57, 52.84, 52.47, 50.17, 43.50, 43.41, 42.41, 37.92, 33.71, 32.27, 31.76, 28.30, 27.63; ESI-MS: *m*/*z* = 708.1 $[M+H]^+$.

*4.1.12.22. 4-(thiazol-2-ylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**42**).* White solid; Yield: 39%; mp: 157–159 °C; Purity: 99.5%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 12.09 (s, 1H, NH), 8.51 (t, 1H, *J* = 6.0 Hz, NH), 8.22 (d, 1H, *J* = 7.5 Hz, NH), 7.80 (t, 1H, *J* = 5.5 Hz, NH), 7.46 (d, 1H, *J* = 2.5 Hz, thiazol-H), 7.42 (d, 1H, *J* = 5.0 Hz, thiazol-H), 7.31–7.15 (m, 8H, Ar-H), 6.74 (d, 1H, *J* = 7.5 Hz, NH), 4.44 (q, 1H, *J* = 7.0 Hz, CH), 4.33 (d, 2H, *J* = 5.0 Hz, CH_2), 4.19 (brs, 1H, CH), 4.04–3.98 (m, 2H, CH_2), 2.86–2.55 (m, 9H, CH + CH_2), 2.10–2.02 (m, 1H, CH_2), 1.95–1.86 (m, 1H, CH_2), 1.81–1.73 (m, 2H, CH_2), 1.56–1.44 (m, 2H, CH_2), 0.80 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 173.40, 172.90, 172.16, 170.66, 158.46, 157.44, 141.84, 138.02, 136.67, 132.34, 129.43, 129.09, 128.92, 128.89, 128.73, 127.50, 126.25, 113.83, 52.85, 52.48, 50.17, 43.50, 43.41, 41.75, 37.93, 33.70, 32.27, 31.76, 28.26, 27.64; ESI-MS: *m*/*z* = 724.1 $[M+H]^+$.

*4.1.12.23. 4-(thiazol-2-yl-methylcarbamoyl)piperidine-1-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl (**43**).* White solid; Yield: 12%; mp: 166–168 °C; Purity: 98.8%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.74 (t, 1H, *J* = 6.0 Hz, NH), 8.52 (t, 1H, *J* = 6.0 Hz, NH), 8.24 (d, 1H, *J* = 8.0 Hz, NH), 7.81 (t, 1H, *J* = 6.0 Hz, NH), 7.71 (d, 1H, *J* = 3.5 Hz, thiazol-H), 7.60 (d, 1H, *J* = 3.0 Hz, thiazol-H), 7.43–7.41 (m, 1H, Ar-H), 7.33–7.27 (m, 5H, Ar-H), 7.26–7.17 (m, 3H, Ar-H), 6.72 (d, 1H, *J* = 7.5 Hz, NH), 4.53 (d, 2H, *J* = 6.0 Hz, CH_2), 4.43 (q, 1H, *J* = 7.5 Hz, CH), 4.32 (d, 2H, *J* = 6.0 Hz, CH_2), 4.19–4.15 (m, 1H, CH), 4.00–3.94 (m, 2H, CH_2), 2.85–2.54 (m, 8H, CH_2), 2.54–2.35 (m, 1H, CH), 2.10–2.03 (m, 1H, CH_2), 1.94–1.87 (m, 1H, CH_2), 1.72–1.63 (m, 2H, CH_2), 1.51–1.47 (m, 2H, CH_2), 0.78 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 174.72, 172.91, 172.16, 170.65, 170.27, 157.43, 142.66, 141.84, 136.68, 132.33, 129.43, 129.07, 128.95, 128.89, 128.74, 127.50, 126.26, 120.32, 52.82, 52.47, 50.16, 43.58, 43.45, 41.99, 37.93, 33.70, 32.27, 31.75, 31.62, 29.44, 28.54, 27.64; ESI-MS: *m*/*z* = 738.3 $[M+H]^+$.

4.1.12.24. 4-Fluorophenyl-carbonyl-Asp(NH-neopentyl)-hPhe-NH-2-chlorobenzyl. White solid; Yield: 71%; mp: 141–143 °C; Purity: 99.4%; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.77 (d, 1H, *J* = 9.0 Hz, NH), 8.53 (t, 1H, *J* = 6.0 Hz, NH), 8.39 (d, 1H, *J* = 10.0 Hz, NH), 7.99–7.90 (m, 3H, NH + Ar-H), 7.43–7.41 (m, 1H, Ar-H), 7.35–7.21 (m, 7H, Ar-H), 7.22–7.14 (m, 3H, Ar-H), 4.82 (q, 1H, *J* = 9.0 Hz, CH), 4.35 (d, 2H, *J* = 7.5 Hz, CH_2), 4.27–4.21 (m, 1H, CH), 2.87–2.71 (m, 4H, CH_2), 2.64–2.53 (m, 2H, CH_2), 2.12–2.04 (m, 1H, CH_2), 1.96–1.88 (m, 1H, CH_2), 0.77 (s, 9H, CH_3); ^{13}C NMR (100 MHz, DMSO): δ = 172.07, 171.75, 170.34, 165.80, 141.77, 136.63, 132.36, 130.91, 130.88, 130.67, 130.58, 129.45, 129.09, 128.96, 128.85, 128.71, 127.51, 126.26, 115.73, 115.52, 52.97, 51.61, 50.20, 42.56, 37.62, 36.70, 34.61, 33.78, 32.26, 31.77, 27.62; ESI-MS: *m*/*z* = 609.3 $[M+H]^+$.

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 72 h, cells were added with MTS at a final concentration of 0.5 mg/mL for 2–4 h. 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 1000 rpm for 5 min and the supernatants are removed. Blood cells were added 100 μL EDTA (5 mM, pH=8.0) and lysed at 4 °C for 60 min, then centrifuged at 6600 × 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. In vivo blood cell proteasome inhibition assay

6-8 weeks normal BALB/c mice were randomized into treatment groups (3 mice per group), and administrated i.v. with vehicle, tested compound and positive control carfilzomib (5 mg/kg) once. After 1, 2, and 24 h, whole blood were collected in K2-EDTA hematocrit tubes via tail vein. 50 μL bloods were washed once with 500 μL PBS, centrifuged with 3300 rpm for 10 min at 4 °C. After discarding the supernants, 100 μL EDTA (5 mM, pH=8.0) were added, and rotated for 60 min at 4 °C. After centrifuging for 10 min with 6000 rpm at 4 °C, the supernants of protein samples were collected. The *in vivo* blood cell proteasome activity assay was followed as the *in vitro* proteasome assay described above.

4.2.5. 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 5594 g 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.

4.3. Molecular modeling

The molecular docking procedure was performed by using GlideSP in Maestro (Schrödinger version 10.7) with the default option [23,24]. The co-crystal structure of proteasome (PDB ID code: 3MG6) was selected as the docking template. For the preparation of protein, the hydrogen atoms were added by using protein preparation Wizard module of Maestro [24], and the OPLS3 force field was employed. For the preparation of ligands, the 3D structures were generated and their energy minimization was performed by using LigPrep [25]. Conformers were generated by using ConfGen [26]. A 30 Å docking grid was generated using centroid of ligand in the 3MG6 crystal structure. Then the ligand was removed and target compound was placed during the molecular docking procedure. Types of interaction of the docked proteasome with ligand were analyzed and then the docking conformations were selected and saved based on calculated glide docking energy score.

Conflict of interest

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

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.12.034.

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