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Research paper

Development of peptide epoxyketones as selective immunoproteasome inhibitors

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ABSTRACT

A series of epoxyketone analogues with varying *N*-caps and P3-configurations were designed, synthesized and evaluated. We found that *D*-Ala in P3 was crucial for β5i selectivity over β5c. Notably, compounds **20j** (β5i IC_{50} = 26.0 nM, 25-fold selectivity) and **20l** (β5i IC_{50} = 25.1 nM, 24-fold selectivity) with the *D*-configuration at P3 were the most selective inhibitors. Although **20j** and **20l** showed only moderate anti-proliferative activity against RPMI-8226 and MM.1S cell lines, based on our experiments, it indicates that the inhibition of β5i alone is not sufficient to exert anticancer effects and may rely on the complementary inhibition of β1i, β5c and β5i. These data further increase our understanding of immunoproteasome inhibitors in hematologic malignancies.

1. Introduction

The ubiquitin proteasome system, discovered in the 1980s, is the major intracellular pathway mediating protein turnover in eukaryotic cells [1]. The system is responsible for the degradation of approximately 80% of all cellular proteins, including misfolded and regulatory proteins [2].

The proteasome is a supramolecular complex composed of a catalytic core particle (CP) and regulatory particles (RPs) [3]. The 20S CP consists of four stacked homologous rings of seven subunits each, two outer α-rings and two inner β-rings, forming a barrel-shaped structure [4,5]. The β-rings act as a proteolytic chamber, containing three catalytic subunits, namely, β1 (caspase-like activity), β2 (trypsin-like activity), and β5 (chymotrypsin-like activity). The function of the α-rings is to maintain a gate through which proteins can enter the proteolytic chamber [6].

Three types of proteasomes exist in vertebrate cells. The constitutive proteasome (cCP) is present in all tissues, whilst the immunoproteasome (iCP) is restricted to hematopoietic cells [7], particularly lymphocytes and monocytes, where it mediates the production of peptides that are subsequently presented by major histocompatibility complex class I (MHC-I) molecules [8,9]. In cortical thymic epithelial cells, the thymoproteasome (tCP) is expressed, which is essential for the optimal positive selection of $CD8^+$ T cells [10,11]. In regards to their proteolytic β-subunits of each, the cCP bears subunits β1c, β2c and β5c, the iCP contains β1i, β2i and β5i, and the tCP holds subunits β1i, β2i and β5t.

Proteasome inhibition has emerged as an anticancer strategy. Three proteasome inhibitors, namely bortezomib [12] (**1**), carfilzomib [13] (**2**), and ixazomib [14] (**3**), have been approved by the Food and Drug Administration (FDA) for the treatment of multiple myeloma (MM) (Fig. 1A).

Despite their remarkable therapeutic efficacy in MM, the use of conventional proteasome inhibitors is often limited because of

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their side effects due to their non-selective inhibition of protein degradation. Thus, selectively targeting proteasome elements would increase efficacy and reduce side effects [15]. Given the relatively limited expression of the immunoproteasome in cells of lymphoid origin, immunoproteasome-specific inhibitors can target hematologic malignancies that originate from lymphoid cells, non-cancer tissues [16]. Inhibiting the immunoproteasome may therefore maintain antimyeloma efficacy whilst reducing toxicity, increasing the therapeutic index.

The immunoproteasome is a constituent of cells of hematopoietic origin, encompassing 30%–90% of the total proteasome in MM cells [17]. Increased immunoproteasome activity has been observed in relapsed/refractory MM [18]. ONX-0914 (**4**, β5i $IC_{50} = 39$ nM, β5c $IC_{50} = 422$ nM, Fig. 1B) was the first described immunoproteasome inhibitor that is selective for β5i [19]. Studies using *in vitro* and *in vivo* MM models showed that the immunoproteasome inhibitor PR-924 (**5**, β5i $IC_{50} = 22$ nM, β5c $IC_{50} = 2900$ nM, Fig. 1B) displayed antitumor activity [20], highlighting the immuno-specific proteasome as a potential treatment strategy.

In this study, to explore the anti-MM activity of immunoproteasome inhibitors, a series of epoxyketone analogues bearing varying *N*-cap and *L*- or *D*-configurations at P3 were designed and synthesized. All compounds were evaluated for their potency against human β5c and β5i. Moreover, inhibitory activity against other subunits and anti-proliferative activity against MM cells were evaluated for selected compounds.

2. Design rationale

In our previous studies, we identified a potent proteasome inhibitor E−83 [21] that can indiscriminately inhibit β5i and β5c to similar levels (β5i $IC_{50} = 21$ nM, β5c $IC_{50} = 12$ nM). ONX-0914 and PR-924 are selective β5i inhibitors. Based on the chemical templates of E−83 and ONX-0914/PR-924, we herein report the discovery of a series of selective β5i inhibitors through fragment-based drug design. We initially incorporated P1, P2 and P3 in PR-924 and the N-cap fragments in E−83. It has been reported by others that a cyclohexyl sidechain in P1 would enhance selectivity for β5i [22]. Consequently, we introduced a cyclohexyl side chain into P1 and further incorporated P2 in ONX-0914, whilst maintaining a cyclohexyl side chain in P1 to design compounds **11a-11f**. Compound **11a** significantly inhibited β5i, but no compounds displayed improved selectivity over ONX-0914. Compound **11f** showed improved selectivity compared to E−83. Further docking studies (Fig. 3) indicated the presence of a 4-(thiazol-2-yl)piperidine *N*-cap in which the *D*-Ala may be too long, which encouraged us to further modify this region. Based on **11a** and **11f**, **20a**-**20n** with short N-caps and the general ONX-0914 formula were designed and synthesized (Fig. 2).

3. Results and discussion

3.1. Chemistry

11a-**11f** were synthesized according to Scheme 1. In route a, **1** was reacted with 2-aminothiazole to produce **2**, which was deprotected with trifluoroacetic acid (TFA) to obtain **3**. In route b, **4a** or **4b** were first reacted with triphosgene to obtain isocyanate intermediates **5a** or **5b**, followed by the addition of **3** to generate the intermediates **6a-6b**, which were hydrolyzed to the corresponding carboxylic derivatives **7a-7b**. In route c, epoxyketone warheads **8a-8b** (the synthesis of which have been reported in the literature [23]) were condensed with Boc-tryptophane or Boc-4-methoxyphenylalanine to generate compounds **9a-9c**, which were deprotected with TFA to generate dipeptide epoxyketone intermediates **10a-10c**. Finally, dipeptide epoxyketone intermediates **10a-10c** were condensed with **7a-7b** to obtain target compounds **11a-11f**.

The synthetic routes for the target compounds **20a-20n** are outlined in Scheme 2. The isocyanate intermediates **5a** or **5b** were reacted with the corresponding amine to generate the intermediates **12a-12d** and **12g-12n**, which were hydrolyzed to produce the carboxylic derivatives **13a-13d** and **13g-13n**, respectively. Carbazole **14** was first reacted with 4-nitrophenyl carbonochloridate to obtain compound **15**, followed by treatment with **5a** or **5b** to produce the intermediates **16a** and **16b**, which were hydrolyzed to produce **17a** and **17b**. The intermediates **13a-13d**, **13g-13h** and **17a-17b** underwent condensation with O-methyl-L-tyrosine methyl ester hydrochloride to afford the dipeptide intermediates **18a-18n**, which were subsequently hydrolyzed to provide the carboxylic intermediates **19a-19n**. The epoxyketone warheads **8a** was condensated with **19a-19n** to generate the target compounds **20a-20n**.

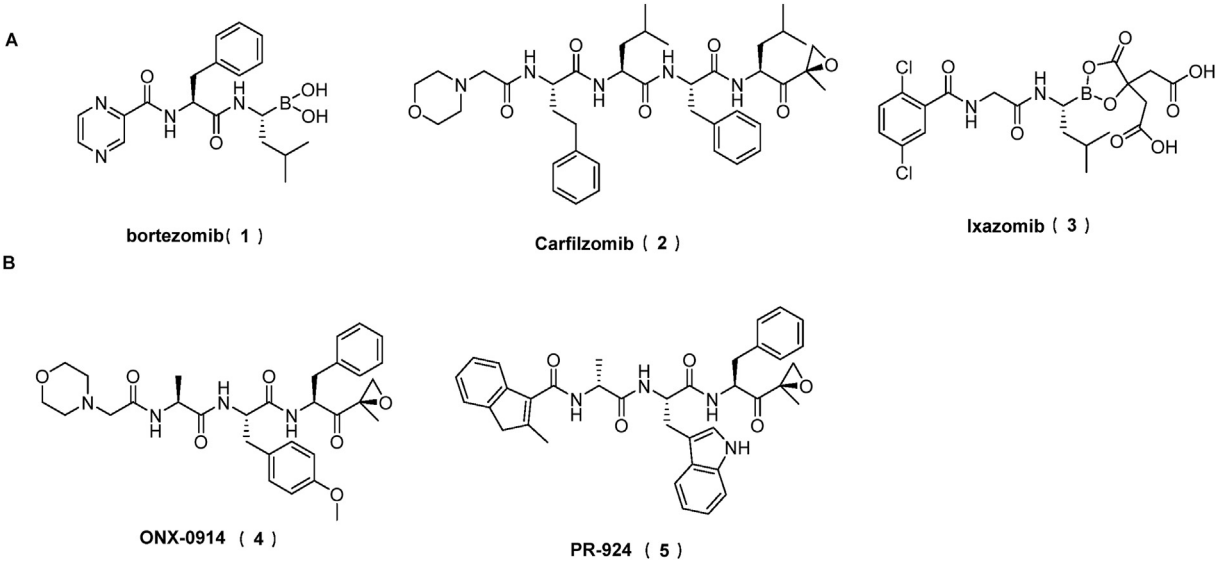


Fig. 1. Proteasome inhibitors. (A) FDA-approved proteasome inhibitors bortezomib, carfilzomib, and ixazomib. (B) Immunoproteasome inhibitors ONX-0914, PR-924.

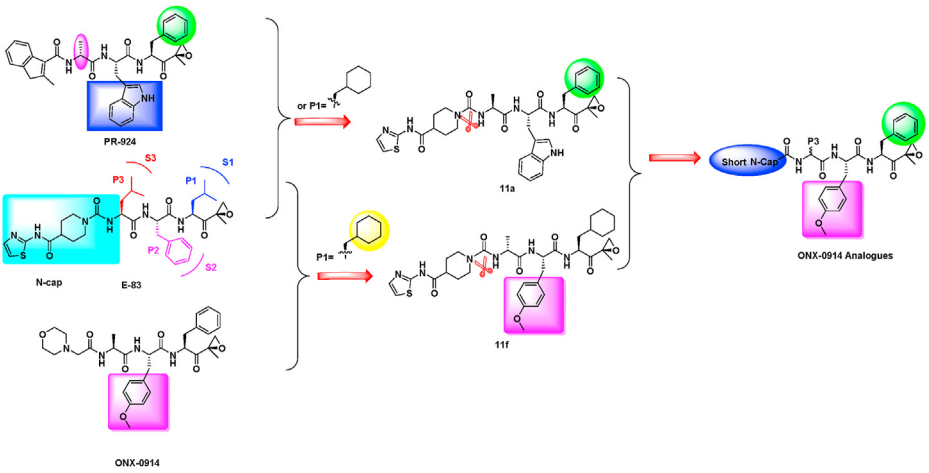
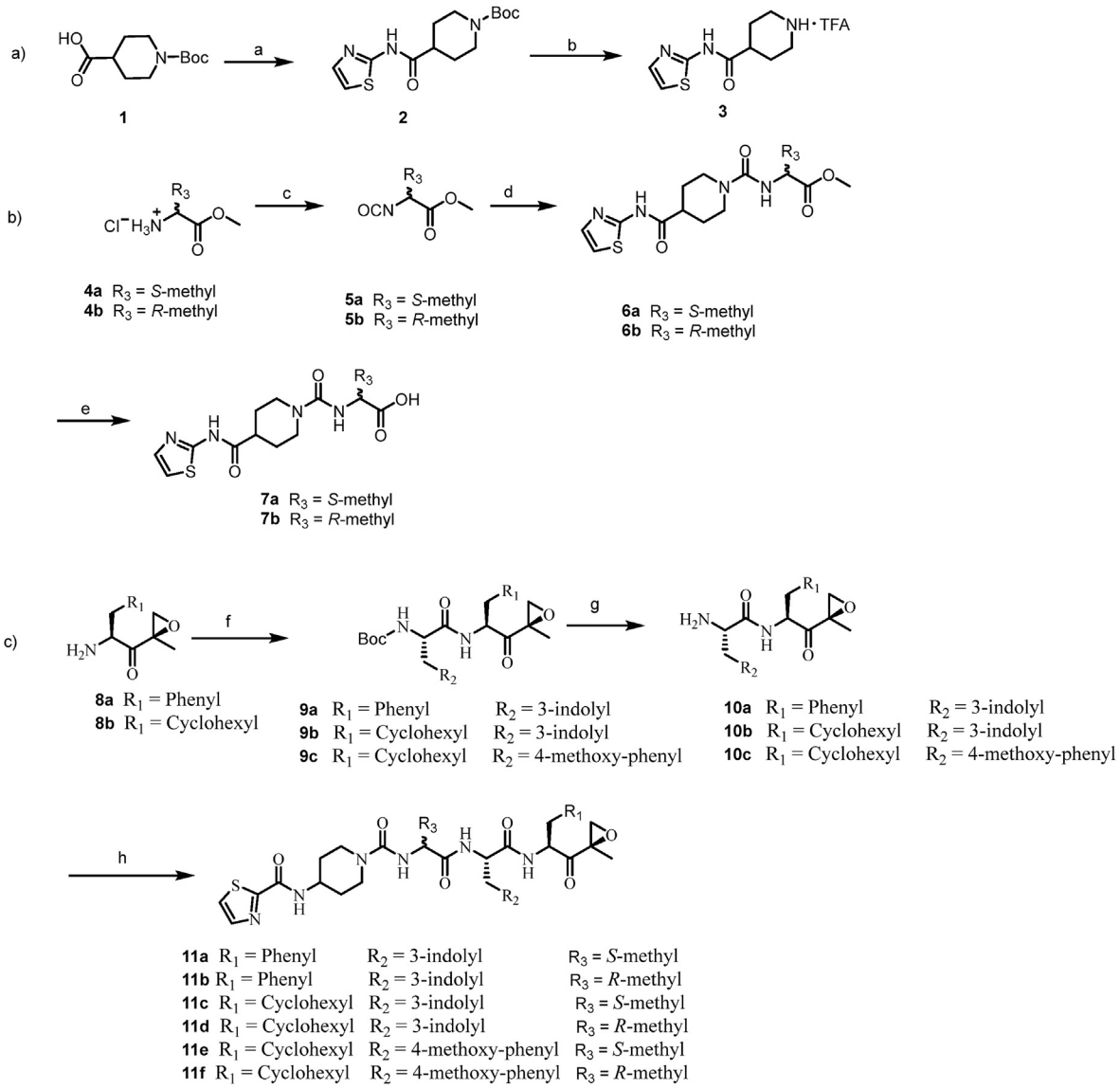
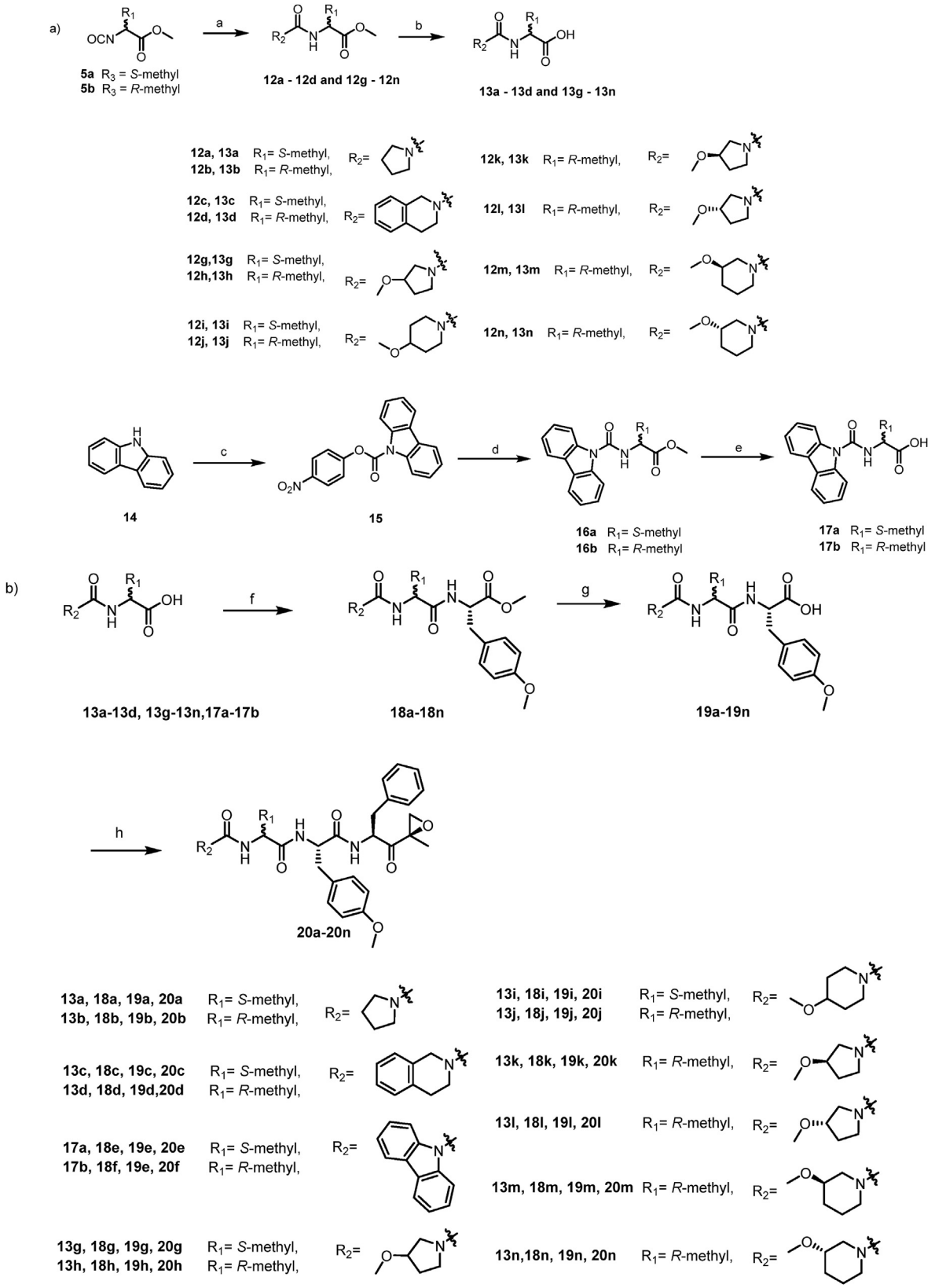


Fig. 2. Design strategy of the selective β5i inhibitors.



Scheme 1. Synthetic routes for the target compounds **11a-11f**. Reagents and conditions: (a) 2-aminothiazole, HOBt, EDCI, DIPEA, DCM, rt, 3 h; (b) TFA, DCM, 0 °C to rt, 3 h; (c) Triphosgene, Saturated $NaHCO_3$, DCM, 0 °C, 15 min; (d) **3**, DIPEA, DCM, rt, 1 h; (e) $LiOH{\cdot}H_2O$, Acetone, H_2O, 6 h; (f) Boc-tryptophane or Boc-4-methoxyphenylalanine, HOBt, EDCI, DIPEA, DCM, rt, 3 h; (g) TFA, DCM, 0 °C, 2 h; (h) **7a-7b**, HOBt, EDCI, DIPEA, DCM, rt, 3 h.



Scheme 2. Synthetic routes for the target compounds **20a-20n**. Reagents and conditions: (a) Corresponding amine, DIPEA, DCM, rt, 1 h; (b) $LiOH \cdot H_2O$, Acetone, H_2O, 6 h; (c) 4-nitrophenyl carbonochloridate, NaH, THF, N_2, overnight; (d) **5a** or **5b**, DIPEA, MeCN, rt, overnight; (e) $LiOH \cdot H_2O$, Acetone, H_2O, -5 °C–0 °C, 30 min; (f) O-methyl-*L*-tyrosine methyl ester hydrochloride, HOBt, EDCI, DIPEA, DCM, rt, 3 h; (g) $LiOH \cdot H_2O$, Acetone, H_2O, −5 °C to 0 °C, 30 min; (h) **8a**, HOBt, EDCI, DIPEA, DCM, rt, overnight.

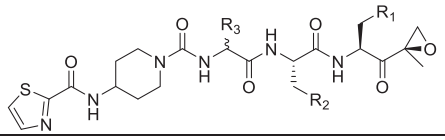
3.2. Structure-activity relationship (SAR) studies

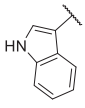
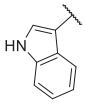
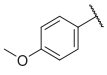
Compounds **11a**-**11f** is presented in Table 1. All compounds were evaluated for their IC_{50} to inhibit β5i and β5c. No compounds displayed improved selectivity over ONX-0914, and all compounds with a *L*-Ala at the R3 position were more potent than compounds with a *D*-Ala (**11a** vs **11b**; **11c** vs **11d**; **11e** vs **11f**), whereas compounds with *D*-Ala were more selective than those with *L*-Ala (**11b** vs **11a**; **11d** vs **11c**; **11f** vs **11e**). When the R2 group was a 3-indolyl, the phenyl group at R1 contributed to the more potent inhibition of β5i compared to the cyclohexyl group (**11a** vs **11c**; **11b** vs **11d**). Analogue **11a** also exhibited higher β5i inhibitory activity with IC_{50} values of 21.1 ± 0.3 nm compared to the positive control ONX-0914 (IC_{50} = 30.5 ± 0.7 nm). We speculated that the phenyl group at R1 contributes to improved β5i activity. When R1 group was a cyclohexyl group, 4-methoxyphenyl substituted analogues were slightly more potent and selective than 3-indolyl substituted compounds (**11e** vs **11c**; **11f** vs **11d**), particularly **11f,** that showed a 3.8-fold preference for β5i, indicating that a 4-methoxyphenyl motif may be beneficial to the selectivity of β5i versus β5c.

To further elucidate the reasons why the potency of compounds with the *D*-configuration in P3 was weaker than the *L*-configuration, we performed docking simulations of compounds **11a**, **11b**, E−83 with proteasome catalytic binding sites (PDB 5L5D, chimeric yeast proteasomes that incorporate key regions of human β5i and the neighboring β6 subunit [27]). As depicted in Fig. 3, modeling of **11b** (*D*-Ala in P3) with β5i/β6 showed a steric clash between the *N*-cap of **11b** and the bottom of the S3* pocket [24], also defined as the β5i S3 pocket formed at the neighboring β6 [25]., This severe steric clash disappeared when an L-Ala was present in P3 (**11a**). With regards to E−83 with an *L*-Leucine in P3, the 4-(thiazol-2-yl) piperidine *N*-cap was a large distance from the S3* pocket, suggesting that such a 4-(thiazol-2-yl)piperidine *N*-cap in compounds with a *D*-configuration may be too long (Fig. 3).

Based on these findings, we shortened the *N*-cap whilst maintaining P1 as a phenyl group, P2 as a 4-methoxyphenyl group

Table 1
Structures and biological data of compounds **11a-11f**.



Cpd.	R_1	R_2	R_3	IC_{50} (nM)[a]		Ratio
				β5i	β5c	β5c/β5i
11a				21.1 ± 0.3	25.6 ± 1.0	1.2
11b				322.5 ± 88.1	468.2 ± 69.6	1.5
11c				245.1 ± 117.5	216.3 ± 71.5	0.9
11d				718.7 ± 347.3	1075.2 ± 548.5	1.5
11e				129.3 ± 13.2	245.4 ± 80.0	1.9
11f				718.0 ± 122.9	2714.0 ± 72.1	3.8
ONX-0914				30.5 ± 0.7	414.8 ± 7.1	13.6

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

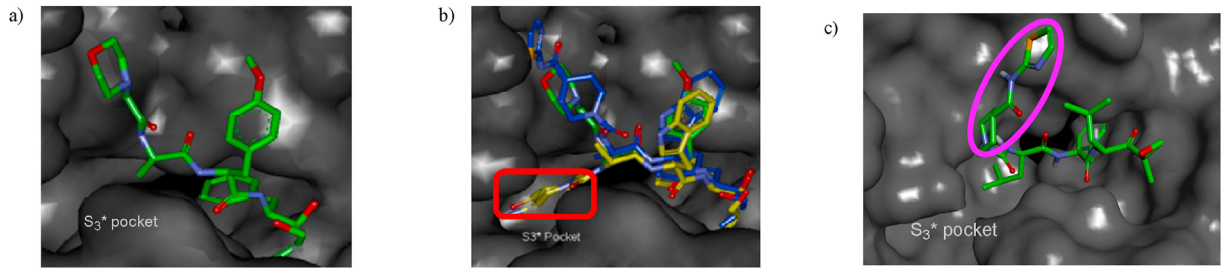


Fig. 3. (a) Complex structure of the human β5i/β6 chimeric proteasome with **ONX-0914** (green) (PDB 5L5D). (b) Overlay of ONX-0914 (green), **11a** (blue) and **11b** (yellow). (c) Predicted binding mode of E−83 into the β5i/β6.

(analogue to ONX-0914) and synthesized analogues of ONX-0914 featuring shorter *N*-cap moieties.

We next synthesized ten analogues of ONX-0914 featuring a pyrrolidine (**20a** and **20b**), tetrahydroisoquinoline (**20c** and **20d**), carbazole (**20e** and **20f**), 3-methoxypyrrolidine (**20g** and **20h**) and 4-methoxypiperidine (**20i** and **20j**) in the N-cap and an *L*-Ala or a *D*-Ala at the P3 position. As shown in Table 2, those with a shortened *N*-cap exhibited moderate to potent β5i inhibitory activity.

When P3 was an *L*-Ala, compounds featuring a hydrophilic *N*-cap (**20g** and **20i**) showed an improved selectivity profile compared to those bearing a hydrophobic *N*-cap (**20a**, **20c** and **20e**). Notably, the chirality of the P3 position had a significant influence on β5i selectivity and potency. When the *N*-cap was a small tetrahydropyrrole group, **20b** (featuring *D*-Ala in P3) showed increased selectivity and enhanced potency for β5i compared to **20a** (bearing *L*-Ala in P3). Although Compound **20b** (β5i IC_{50} = 62.9 nM) was slightly less active than the positive compound ONX-0914, it was more selective than **ONX-0914** for β5i over β5c (21-fold vs 7-fold). Compound **20d** with a *D*-Ala in P3 also showed improved selectivity than *L*-Ala in the P3 analogue **20c**, showing a 12-fold preference for β5i. Next, we expanded the *N*-cap and synthesized carbazole analogues **20e** and **20f**. Although **20f** exhibited high affinity, its selectivity was lower than that of compounds with a smaller group in the *N*-cap (**20b** and **20d**). This indicates that a bulky hydrophobic *N*-cap was not beneficial for β5i selectivity. The most prominent improvement in selectivity was noticed for **20h** and **20j** which displayed 25-fold selectivity for β5i over β5c. And **20j** (β5i IC_{50} = 26.0 nM) was as potent as ONX-0914 (β5i IC_{50} = 28.2 nM).

We concluded that the selectivity of compounds with P3-*D* (**20b**, **20d**, **20f**, **20h** and **20j**) were higher than those with P3-*L* (**20a**, **20c**, **20e**, **20g** and **20i**) while remaining or even enhancing the binding affinity toward β5i.

To determine the optimal moiety, we selected **20h** and **20j** (P3-*D*-Ala) for further optimization. As a result of investigating the substitution pattern of the methoxyl group, a decrease in selectivity and inhibitory activity was observed when the 4-methoxyl group was replaced with the 3-methoxyl group at the piperidine (**20m** or **20n** versus **20j**). Considering that **20h** was a mixture (a mixture of compounds featuring *R*-3-methoxy-pyrrolidine *N*-cap and *S*-3-methoxy-pyrrolidine *N*-cap), we further took them apart to examined the activity and selectivity of each isomer. Compound **20k** and **20l** were similar in selectivity, but **20l** with *S*-3-methoxy-pyrrolidine showed improved potency than **20k** featuring the *R* isomer.

3.3. Inhibitory activity against β1c/β1i/β2c/β2i

To establish the IC_{50} values more accurately and also to obtain insights into inhibitory activities against β1c/β1i/β2c/β2i, we selected four potent inhibitors that were comparable to ONX-0914 against β5i, with high β5i-selectivity (**20l**, **20j**), moderate β5i-selectivity (**20m**), and weak β5i-selectivity (**11a**). These were assayed with ONX-0914 as the positive control compound. These data are shown in Table 3.

Inhibition profiling studies revealed in addition to potent inhibitory activity against β5i, ONX-0914 was moderately potent against β5c and β1i with IC_{50} values of 196.7 nM and 267.1 nM, respectively. Apart from β5i and β5c, compounds **11a** also demonstrated moderate potency against β1i with a IC_{50} of 154.0 nM, whereas **20m**, **20j**, **20l** minimally affected β1i, and IC_{50} values of all the compounds against other subunits demonstrated >4 μM.

3.4. Anti-proliferative activity against cancer cell lines

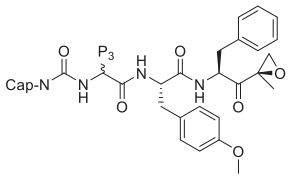
To investigate whether targetting β5i selectively is efficacious for the treatment of hematologic malignancies, **20l**, **20j**, **20m**, **11a** were also evaluated in human multiple myeloma cells (RMPI-8226 and MM.1S) to assess their anti-proliferative activity *in vitro*. Assays were validated using ONX-0914 as a reference compound. The results are summarized in Table 4.

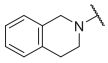
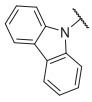
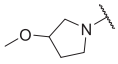
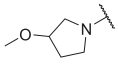
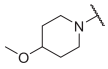
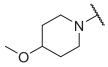
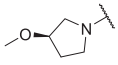
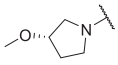
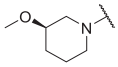
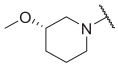
Unexpectedly, **20j** (25-fold selectivity) and **20l** (24-fold selectivity), the most selective compounds over β5c showed only moderate cell growth inhibitory effects against RPMI-8226 and MM.1S cells, whereas the growth inhibitory effects of the less selective compound **11a** (1-fold selectivity) was comparable to ONX-0914 against RPMI-8226 cells, with IC_{50} values of 0.11 μM and 0.15μM, respectively. And compound **20m** which exhibited 8-fold selectivity over β5c displayed higher anti-proliferative activity than **20l** and **20j** and lower activity than ONX-0914.

We next analyzed the possible causes leading to the apparent discrepancy between activities of different subunits and anti-proliferative activity against MM cell lines. As shown in Table 3, ONX-0914 strongly inhibited β5i and was moderately potent against β5c and β1i. Although compound **20m** showed comparable inhibitory activities to ONX-0914 against β5c and β5i, its anti-proliferative activities against two human MM cell lines were weaker 3−5 fold than ONX-0914, which be explained by its poor inhibitory activity against β1i. Compound **11a** with strong anti-proliferative activity showed similar inhibitory activity against β1i to ONX-0914. Only moderate inhibitory activities were observed against β5c for compounds **20j** and **20l,** which demonstrated micromolar inhibitory potency against β1i. This may explain why **20j** and **20l** showed weak cell growth inhibitory effects. Based on these data, we conclude that the inhibition of β5i alone may be insufficient to exert the anticancer effects and the co-inhibition of β1i, β5c and β5i may be required for its anti-leukemic effects.

These results were consistent with Niewerth et al. who assessed the anti-leukemic activity of PR-924 against the MM cell line RPMI-8226. They showed that PR-924 displayed cytotoxic activity in the micromolar range [26]. And at higher concentrations of 1−10 μM,

Table 2
Structures and biological data of compounds **20a**-**20n.**



Cpd.	P_3	*N*-Cap	IC_{50} (nM)[a]		Ratio
			*β*5i	*β*5c	*β*5c/*β*5i
20a			246.3 ± 45.2	498.3 ± 65.3	2.0
20b			62.9 ± 9.3	1332.5 ± 224.2	21.2
20c			37.8 ± 3.0	69.7 ± 30.1	1.8
20d			80.7 ± 11.7	959.5 ± 304.8	11.9
20e			61.0 ± 13.7	35.5 ± 3.8	0.6
20f			38.2 ± 15.1	71.7 ± 14.3	1.9
20g			56.8 ± 3.4	293.5 ± 49.8	5.2
20h			44.5 ± 0.7	1106.6 ± 338.6	24.8
20i			18.9 ± 4.4	74.0 ± 7.2	3.9
20j			26.0 ± 6.28	647.1 ± 121.0	24.9
20k			66.0 ± 33.8	1483.5 ± 143.5	22.5
20l			25.1 ± 3.6	606.4 ± 212.3	24.1
20m			29.9 ± 5.1	244.1 ± 45.3	8.2
20n			37.7 ± 10.8	162.6 ± 89.4	4.3
ONX-0914			28.2 ± 9.2	196.7 ± 77.0	7.0

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

Table 3
IC_{50} values of inhibitors against *β*1c/*β*1i/*β*2c/*β*2i.

Cpd	IC_{50}(nm)						
	*β*1c	*β*1i	*β*2c	*β*2i	*β*5c	*β*5i	Ratio (*β*5c/*β*5i)
11a	4355.5 ± 696.5	154.0 ± 16.1	>10000	>10000	25.6 ± 1.0	21.1 ± 0.3	1.2
20m	>10000	4273.0 ± 14.1	>10000	>10000	244.1 ± 45.3	29.9 ± 5.1	8.2
20l	>10000	5770.0 ± 1074.8	>10000	>10000	606.4 ± 212.3	25.1 ± 3.6	24.1
20j	>10000	>10000	>10000	>10000	647.1 ± 121.0	26.0 ± 6.28	24.9
ONX-0914	>10000	267.1 ± 73.8	1603.5 ± 208.6	1697.00 ± 287.1	196.7 ± 77.0	28.2 ± 9.2	7.0

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

Table 4
IC_{50} values of inhibitors against RPMI-8226 and MM.1S cells.

Cpd	Cytotoxicity (IC_{50}, μM)[a]	
	RPMI-8226	MM.1S
11a	0.11 ± 0.02	0.11 ± 0.02
20m	0.68 ± 0.08	0.22 ± 0.24
20l	1.88 ± 0.38	1.37 ± 0.35
20j	1.92 ± 0.26	1.79 ± 0.39
ONX-0914	0.15 ± 0.01	0.06 ± 0.01

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

near complete inhibition of β5c and β1i catalytic activities was observed, which indicated β5i inhibition alone is not sufficient to elicit an anti-leukemic effect, with PR-924 paralleling a concomitant inhibition of β1i, β5c and β5i at drug concentrations >1 μM [26].

3.5. Molecular docking analysis

Docking is an effective method to propose the binding interactions between a ligand and the receptor. In this study, we have chosen the most potent and selective compound **20l** against β5i/β6 for molecular modeling using Discovery Studio (version 2.1). As depicted in Fig. 4, The proposed binding pose of compound **20l** in complex with β5i/β6 has four important H-bonds. Two of these H-bonds are located between the carbonyl group of epoxyketone warhead and Ser 21 and Gly 23 amino acid residues. The third H-bond of compound **20l** is observed between the NH of the amide group of phenylalanine in P1 and the carbonyl group of Ser 21 amino acid. And the last H-bond was formed between the carbonyl group of N-cap and the hydroxyl group of Ser 27. These H-bonds demonstrate that the structure of the ligand is stabilized by the three key amino acids.

4. Conclusions

In summary, this study describes a series of epoxyketone inhibitors with varying *N*-caps and P3 configurations. SARs indicated that the *D*-Ala in the P3 residue was crucial to increasing the selectivity for β5i over β5c whilst maintaining or enhancing β5i-potency. Analogues **20j** (β5i IC_{50} = 26.0 nM, 25-fold) and **20l** (β5i IC_{50} = 25.1 nM, 24-fold) with *D*-Ala at the P3 site were the most selective inhibitors, which showed higher selectivity than ONX-0914. Docking studies on compound **20l** revealed key interactions with the catalytic binding sites. Although **20j** and **20l** showed only moderate anti-proliferative activity against RPMI-8226 and MM.1S cell lines, based on our experiments, the inhibition of β5i alone was insufficient to exert anticancer effects, which likely rely on complementary inhibition of β1i, β5c and β5i. Our results will further increase the understanding of the immunoproteasome inhibitors in hematologic malignancies.

5. Experimental section

5.1. Chemistry

5.1.1. General methods

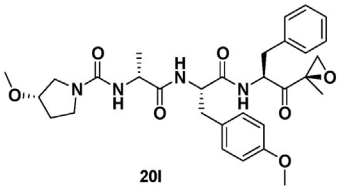
All reagents and solvents were purchased from commercial suppliers and used without further purification unless otherwise indicated. Flash-column chromatography was performed using silica gel (200–300 mesh). ^1H and ^{13}C NMR spectra were recorded on a Bruker AV-400 (400 MHz) or AV-500 (500 MHz) spectrometer. Chemical shifts (*d*) are given in ppm (δ) relative to Chloroform-*d* or DMSO-d_6 as internal standard. Melting points were determined using a Buchi B-540 capillary melting point apparatus. LC–MS analysis was performed on a Shimadzu LCMS-2020 mass spectrometer with mobile phases as methanol and water containing 0.1% formic acid. HPLC analysis was performed using an Agilent 1260 Series system with a COSMOSIL 5C18-MS-II (4.6 mm × 250 mm) column and detected at 220 nm wavelength. The details can be found in supplementary data. All tested compounds are >95% pure.

5.1.2. General procedure A for the synthesis of intermediates 6a-6b, 12a-12d & 12g-12n

Alanine methyl ester hydrochloride **4a** or **4b** (1.0 equiv) was dissolved in DCM (500 mg/25 mL) and saturated $NaHCO_3$ (V_{DCM}:$V_{saturated\ NaHCO3}$ = 1:1) was added. The mixture was then cooled to 0 °C, and triphosgene was added. The mixture was stirred at 0 °C for 15min, after which the organic layer was separated and washed with brine (1 ×), dried over Na_2SO_4. The organic layer was then cooled to 0 °C and corresponding amine (0.8 equiv) and DIPEA (5 equiv) were added. The reaction mixture was allowed stirred at room temperature for 3 h. Then the mixture was washed with brine (1 ×) and dried over Na_2SO_4. The organic layer was evaporated in vacuo and the crude product was purified by flash-column chromatography on silica gel.

5.1.2.1. Methyl (4-(thiazol-2-ylcarbamoyl)piperidine-1-carbonyl)-L-alaninate (6a). Compound **6a** was obtained by the general procedure A from commercial intermediate **3** and **5a.** Yield: 83%; White solid; ^1H NMR (500 MHz, DMSO-d_6) δ 12.07 (s, 1H), 7.46 (d, *J* = 3.0 Hz, 1H), 7.19 (d, *J* = 2.5 Hz, 1H), 6.74 (d, *J* = 5.8 Hz, 1H), 4.18–4.11 (m, 1H), 4.03 (m, 2H), 3.61 (s, 3H), 2.69 (m, 3H), 1.77 (d, *J* = 12.4 Hz, 2H), 1.57–1.43 (m, 2H), 1.28 (d, *J* = 7.0 Hz, 3H); ESI-MS: *m/z* = 341 $[M+H]^+$.

5.1.2.2. Methyl (4-(thiazol-2-ylcarbamoyl)piperidine-1-carbonyl)-D-alaninate (6b). Compound **6b** was obtained by the general procedure



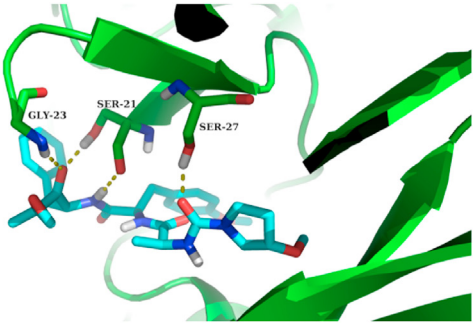


Fig. 4. Predicted binding mode of **20l** into the β5i/β6.

A from commercial intermediate **3** and **5b.** Yield: 85%; White solid; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.38 (d, J = 3.5 Hz, 1H), 6.97 (d, J = 3.5 Hz, 1H), 4.44 (q, J = 7.0 Hz, 1H), 4.06–3.96 (m, 2H), 3.73 (s, 3H), 2.90 (m, 2H), 2.65–2.56 (m, 1H), 1.92 (m, 2H), 1.83–1.74 (m, 2H), 1.38 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 341 $[M+H]^+$.

5.1.2.3. Methyl (pyrrolidine-1-carbonyl)-L-alaninate (12a). Compound **12a** was obtained by the general procedure A from commercial pyrrolidine and **5a**. Yield: 81%; Yellowish-white solid; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.74 (brs, 1H), 4.57–4.49 (m, 1H), 3.74 (s, 3H), 3.42–3.28 (m, 4H), 1.95–1.85 (m, 4H), 1.40 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 201 $[M + H]^+$.

5.1.2.4. Methyl (pyrrolidine-1-carbonyl)-D-alaninate (12b). Compound **12b** was obtained by the general procedure A from commercial pyrrolidine and **5b**. Yield: 79%; white solid; mp = 91.2–92.0 °C; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.74 (s, 1H), 4.56–4.48 (m, 1H), 3.73 (s, 3H), 3.40–3.29 (m, 4H), 1.93–1.85 (m, 4H), 1.39 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 201 $[M + H]^+$.

5.1.2.5. Methyl (1,2,3,4-tetrahydroisoquinoline-2-carbonyl)-L-alaninate (12c). Compound **12c** was obtained by the general procedure A from commercial 1,2,3,4-tetrahydroisoquinoline and **5a**. Yield: 80%; Colorless oil; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.22–7.10 (m, 4H), 5.09 (s, 1H), 4.56 (m, 3H), 3.75 (s, 3H), 3.62 (m, 2H), 2.88 (t, J = 5.6 Hz, 2H), 1.43 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 263 $[M + H]^+$

5.1.2.6. Methyl (1,2,3,4-tetrahydroisoquinoline-2-carbonyl)-D-alaninate (12d). Compound **12d** was obtained by the general procedure A from commercial 1,2,3,4-tetrahydroisoquinoline and **5b**. Yield: 82%; Colorless oil; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.23–7.08 (m, 4H), 5.08 (d, J = 7.0 Hz, 1H), 4.62–4.51 (m, 3H), 3.75 (s, 3H), 3.63 (m, 2H), 2.88 (t, J = 5.6 Hz, 2H), 1.43 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 263 $[M + H]^+$.

5.1.2.7. Methyl(3-methoxypyrrolidine-1-carbonyl)-L-alaninate (12g). Compound **12g** was obtained by the general procedure A from 3-methoxypyrrolidine and **5a**. Yield: 82%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.79 (s, 1H), 4.50 (q, J = 7.0 Hz, 1H), 3.95 (m, 1H), 3.72 (m, 3H), 3.47–3.38 (m, 4H), 3.30 (m, 3H), 2.04 (m, 1H), 1.99–1.93 (m, 1H), 1.38 (m, 3H); ESI-MS: m/z = 231 $[M + H]^+$.

5.1.2.8. Methyl (3-methoxypyrrolidine-1-carbonyl)-D-alaninate (12h). Compound **12h** was obtained by the general procedure A from 3-methoxypyrrolidine and **5b**. Yield: 85%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.77 (s, 2H), 4.51 (d, J = 6.0 Hz, 2H), 4.00–3.93 (m, 2H), 3.73 (s, 6H), 3.48–3.38 (m, 8H), 3.32 (s, 3H, isomer), 3.31 (s, 3H, isomer), 2.09–2.03 (m, 2H), 1.98 (m, 2H), 1.39 (d, J = 7.2 Hz, 6H); ESI-MS: m/z = 231 $[M + H]^+$.

5.1.2.9. Methyl (4-methoxypiperidine-1-carbonyl)-L-alaninate (12i). Compound **12i** was obtained by the general procedure A from 4-methoxypiperidine and **5a**. Yield: 79%; Colorless oil; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.05 (brs, 1H), 4.52–4.43 (m, 1H), 3.74 (s, 3H), 3.70–3.59 (m, 2H), 3.41–3.36 (m, 1H), 3.34 (s, 3H), 3.14 (m, 2H), 1.92–1.81 (m, 2H), 1.62–1.51 (m, 2H), 1.39 (d, J = 7.2 Hz, 3H); ESI-MS: m/z = 245 $[M + H]^+$.

5.1.2.10. Methyl (4-methoxypiperidine-1-carbonyl)-D-alaninate (12j). Compound **12j** was obtained by the general procedure A from 4-methoxypiperidine and **5b**. Yield: 82%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.01 (d, J = 6.5 Hz, 1H), 4.48 (m, 1H), 3.73 (s, 3H), 3.69–3.59 (m, 2H), 3.38 (m, 1H), 3.34 (s, 3H), 3.13 (m, 2H), 1.85 (m, 2H), 1.60–1.51 (m, 2H), 1.38 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 245 $[M + H]^+$.

5.1.2.11. Methyl ((R)-3-methoxypyrrolidine-1-carbonyl)-D-alaninate (12k). Compound **12k** was obtained by the general procedure A from (*R*)-3-methoxypyrrolidine and **5b**. Yield: 75%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.79 (d, J = 6.0 Hz, 1H), 4.52 (p, J = 7.0 Hz, 1H), 3.99–3.95 (m, 1H), 3.74 (s, 3H), 3.48–3.42 (m, 4H), 3.32 (s, 3H), 2.09–2.03 (m, 1H), 2.01–1.93 (m, 1H), 1.40 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 231 $[M + H]^+$.

5.1.2.12. Methyl ((S)-3-methoxypyrrolidine-1-carbonyl)-D-alaninate (12l). Compound **12l** was obtained by the general procedure A from (S)-3-methoxypyrrolidine and **5b**. Yield: 78%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.78 (d, J = 6.0 Hz, 1H), 4.56–4.47 (m, 1H), 3.99–3.95 (m, 1H), 3.74 (s, 3H), 3.51 (d, J = 11.0 Hz, 1H), 3.46–3.39 (m, 3H), 3.32 (s, 3H), 2.12–2.04 (m, 1H), 2.03–1.96 (m, 1H), 1.40 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 231 $[M + H]^+$.

5.1.2.13. Methyl ((R)-3-methoxypiperidine-1-carbonyl)-D-alaninate (12 m). Compound **12m** was obtained by the general procedure A from (*R*)-3-methoxypiperidine and **5b**. Yield: 75%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.04 (d, J = 6.0 Hz, 1H), 4.48 (p, J = 7.0 Hz, 1H), 3.81 (dd, J = 13.0, 3.5 Hz, 1H), 3.74 (s, 3H), 3.52–3.46 (m, 1H), 3.37 (s, 3H), 3.28–3.23 (m, 1H), 3.13–3.05 (m, 1H), 2.99 (dd, J = 13.0, 8.0 Hz, 1H), 1.99–1.93 (m, 1H), 1.79–1.73 (m, 1H), 1.53–1.44 (m, 2H), 1.39 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 245 $[M + H]^+$.

5.1.2.14. Methyl ((S)-3-methoxypiperidine-1-carbonyl)-D-alaninate (12n). Compound **12n** was obtained by the general procedure A from (S)-3-methoxypiperidine and **5b**. Yield: 73%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 5.09 (s, 1H), 4.51–4.41 (m, 1H), 3.75–3.71 (m, 4H), 3.50–3.43 (m, 1H), 3.37 (s, 3H), 3.28–3.21 (m, 1H), 3.16–3.06 (m, 2H), 1.96–1.90 (m, 1H), 1.76 (m, 1H), 1.57–1.46 (m, 2H), 1.39 (d, J = 7.0 Hz, 3H); ESI-MS: m/z = 245 $[M + H]^+$.

5.1.3. Methyl (9H-carbazole-9-carbonyl)-L-alaninate (16a)

To a solution of carbazole **14** (669 mg, 4.0 mmol) in anhydrous THF (20 mL) was added NaH (192 mg, 4.8 mmol) at 0 °C. The mixture was stirred under argon for 30 min at the same temperature. 4-nitrophenyl carbonochloridate (947 mg, 4.7 mmol) was added to the mixture and the mixture was stirred under argon at 0 °C for 1 h. After that, the reaction solution was allowed to warm to room temperature and stirred overnight. The volatiles were removed under reduced pressure. The crude product was purified by flash-column chromatography to provided **15**. Yellow soild (797 mg, 60%); mp = 181.9–182.0 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.42 (d, J = 9.0 Hz, 2H), 8.29 (d, J = 8.5 Hz, 2H), 8.25 (d, J = 7.5 Hz, 2H), 7.85 (d, J = 9.0 Hz, 2H), 7.60–7.54 (m, 2H), 7.48 (m, 2H). ESI-MS: m/z = 333 $[M + H]^+$.

To a solution of **15** (332 mg, 1.0 mmol) in THF (10 mL) was added **5a** (1116 mg, 8.0 mmol) followed by DIPEA (1.36 mL, 8.0 mmol). The mixture was stirred at room temperature overnight. The solvent was evaporated in vacuum, and the residue was diluted with EtOAc (10 mL). The organic layer was washed with saturated $NaHCO_3$ (10 mL × 2), brine (10 mL × 1), and dried over Na_2SO_4. Purification by flash-column chromatography. White soild (130 mg, 44%); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.12 (d, J = 8.5 Hz, 2H), 8.04 (d, J = 7.5 Hz, 2H), 7.50 (ddd, J = 8.5, 7.5, 1.0 Hz, 2H), 7.39–7.33 (m, 2H), 6.42 (d, J = 7.0 Hz, 1H), 4.88 (m, 1H), 3.86 (s, 3H), 1.64 (d, J = 7.0 Hz, 3H). ESI-MS: m/z = 297 $[M + H]^+$.

5.1.4. Methyl (9H-carbazole-9-carbonyl)-D-alaninate (16b)

To a solution of **15** (332 mg, 1.0 mmol) in THF (10 mL) was added **5b** (1116 mg, 8.0 mmol) followed by DIPEA (1.36 mL, 8.0 mmol). The mixture was stirred at room temperature overnight. The solvent was evaporated in vacuum, and the residue was diluted with EtOAc (10 mL). The organic layer was washed with saturated $NaHCO_3$

(10 mL × 2), brine (10 mL × 1), and dried over Na_2SO_4. Purification by flash-column chromatography. White soild (150 mg, 51%); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.12 (d, *J* = 8.5 Hz, 2H), 8.03 (d, *J* = 7.5 Hz, 2H), 7.53–7.47 (m, 2H), 7.39–7.33 (m, 2H), 6.42 (d, *J* = 7.0 Hz, 1H), 4.88 (m, 1H), 1.64 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, CDCl3) δ 173.59, 151.97, 138.28, 127.10, 125.24, 122.49, 120.17, 113.77, 52.82, 49.53, 18.93. ESI-MS: *m/z* = 297 $[M + H]^+$.

5.1.5. General procedure B for the synthesis of carboxylic derivatives

Compounds (1.0 equiv) were dissolved in acetone and at −10 °C added 0.3 N LiOH (1.2 equiv) dropwise. The solution was stirred at −0 °C for 30 min. The mixture was then acidified to pH = 2–3 with 3 N HCl and extracted with EtOAc (3 ×) or *N*-butanol (3 ×) (if necessary). The organic layer was dried over Na_2SO_4, evaporated in vacuo to yield the crude product without further purification.

5.1.5.1. Carboxylic derivatives 7a-7b, 13a-13d, 13g-13n, 17a-17b and 19a-19n. Compound **7a-7b**, **13a-13d**, **13g-13n and 19a-19n** were obtained by the general procedure B. The crude product were used in the next step without further purification.

5.1.6. N-(thiazol-2-yl)piperidine-4-carboxamide-trifluoroacetate (3)

Compound **2** (5.0 g, 16.1 mmol) was dissolved in DCM and cooled to 0 °C, added dropwise trifluoroacetic acid (16 mL). The mixture was allowed to warm to room temperature and stirred for 3 h. After completion of the reaction, the solvent was evaporated under reduced pressure. Then the THF was added to the crude product, precipitating 3.6 g white solid (70%); mp:188-189 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.26 (s, 1H), 8.62 (s, 1H), 8.37 (s, 1H), 7.48 (d, *J* = 3.5 Hz, 1H), 7.23 (d, *J* = 3.5 Hz, 1H), 3.33 (d, *J* = 13.0 Hz, 2H), 2.99–2.88 (m, 2H), 2.81–3.75 (m, 1H), 2.02–1.94 (m, 2H), 1.87–1.74 (m, 2H).; ESI-MS: *m/z* = 212 $[M+H]^+$.

5.1.7. General procedure C for the removal of t-butyloxycarbonyl (Boc) protecting group on dipeptide epoxyketone fragment 10a, 10b and 10c

The dipeptide epoxyketone fragment was dissolved in DCM and cooled to 0 °C, added dropwise trifluoroacetic acid. The mixture was stirred for 3 h at 0 °C, the solvent was evaporated under reduced pressure to get the light yellow oil and the crude product were used in the next step without further purification.

5.1.8. General procedure D for the synthesis of the intermediates 2, 9a-9c, 18a-18n, 11a-11f and 20a-20n

The crude acid (1.0 equiv) was dissolved in DCM and cooled to 0 °C. The solution was then treated with HOBt (1.2 equiv), EDCI (1.8 equiv), corresponding amine or amino acid (1.2 equiv) and DIPEA (3.0 equiv). The mixture was allowed to warm to room temperature and stirred for 3 h. After completion of the reaction, the mixture was washed with 1 N HCl (2 ×), saturated $NaHCO_3$ (2 ×), and brine (2 ×), and dried over Na_2SO_4. The organic layer was evaporated in vacuo and the crude product was purified by flash-column chromatography on silica gel.

5.1.8.1. Tert-butyl 4-(thiazol-2-ylcarbamoyl)piperidine-1-carboxylate (2). Compound **2** was obtained by the general procedure D from **1** and 2-aminothiazole. Yield:89%; white solid; mp:192-194 °C;^{1}H NMR (500 MHz, Chloroform-*d*) δ 12.04 (brs, 1H), 7.41 (d, *J* = 3.5 Hz, 1H), 7.03 (d, *J* = 3.5 Hz, 1H), 4.18 (s, 2H), 2.91–2.76 (m, 2H), 2.63 (tt, *J* = 11.0, 4.0 Hz, 1H), 1.91 (d, *J* = 11.5 Hz, 2H), 1.86–1.76 (m, 2H), 1.47 (s, 9H); ESI-MS: *m/z* = 312 $[M+H]^+$.

5.1.8.2. Tert-butyl ((S)-3-(1H-indol-3-yl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)carbamate (9a). Compound **9a** was obtained by the general procedure D from Phe-epoxyketone and (*tert*-butoxycarbonyl)-L-tryptophan. Yield: 60%; yellow soild; mp:83–85 °C;^{1}H NMR (500 MHz, DMSO-d_6) δ 10.79 (s, 1H), 8.28 (d, *J* = 7.5 Hz, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.26 (m, 6H), 7.05 (t, *J* = 7.5 Hz, 1H), 7.01 (s, 1H), 6.97 (t, *J* = 7.5 Hz, 1H), 6.69 (d, *J* = 8.5 Hz, 1H), 4.64–4.59 (m, 1H), 4.22–4.15 (m, 1H), 3.16 (d, *J* = 5.0 Hz, 1H), 2.95 (m, 3H), 2.80 (m, 1H), 2.73 (m, 1H), 1.36 (s, 3H), 1.29 (s, 9H); ESI-MS: *m/z* = 492 $[M+H]^+$.

5.1.8.3. Tert-butyl ((S)-1-(((S)-3-cyclohexyl-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)amino)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)carbamate (9b). Compound **9b** was obtained by the general procedure D from cyclohexyl-epoxyketone and (*tert*-butoxycarbonyl)-L-tryptophan. Yield: 61%; yellow soild; mp:151–152 °C;^{1}H NMR (500 MHz, DMSO-d_6) δ 10.81 (s, 1H), 8.15 (d, *J* = 7.5 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.31 (d, *J* = 8.0 Hz, 1H), 7.10 (s, 1H), 7.05 (t, *J* = 7.5 Hz, 1H), 6.97 (t, *J* = 7.5 Hz, 1H), 6.71 (d, *J* = 8.5 Hz, 1H), 4.45 (m, 1H), 4.22 (m, 1H), 3.15 (d, *J* = 5.0 Hz, 1H), 3.05–2.94 (m, 2H), 2.84 (dd, *J* = 14.5, 9.5 Hz, 1H), 1.75–1.54 (m, 5H), 1.40 (m, 4H), 1.30 (s, 9H), 1.22–1.08 (m, 5H), 0.99–0.90 (m, 1H), 0.82 (m, 1H); ESI-MS: *m/z* = 498 [M+H]+.

5.1.8.4. Tert-butyl ((S)-1-(((S)-3-cyclohexyl-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)amino)-3-(4-methoxyphenyl)-1-oxopropan-2-yl)carbamate (9c). Compound **9c** was obtained by the general procedure D from cyclohexyl-epoxyketone and (*S*)-2-((*tert*-butoxycarbonyl)amino)-3-(4-Methoxyphenyl)propanoic acid. Yield: 63%; white soild; mp: 153–155 °C;^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.12 (d, *J* = 8.0 Hz, 2H), 6.83 (d, *J* = 8.5 Hz, 2H), 6.12 (d, *J* = 7.5 Hz, 1H), 4.95 (d, *J* = 5.0 Hz, 1H), 4.60–4.55 (m, 1H), 4.28 (m, 1H), 3.79 (s, 3H), 3.25 (d, *J* = 2.5 Hz, 1H), 3.03 (dd, *J* = 14.0, 6.5 Hz, 1H), 2.93 (dd, *J* = 14.0, 7.0 Hz, 1H), 2.88 (d, *J* = 5.0 Hz, 1H), 1.81 (d, *J* = 12.5 Hz, 1H), 1.73–1.52 (m, 6H), 1.49 (s, 3H), 1.42 (s, 9H), 1.22–1.09 (m, 5H), 0.89 (m, 2H); ESI-MS: *m/z* = 489 $[M+H]^+$.

5.1.8.5. Methyl(S)-3-(4-methoxyphenyl)-2-((S)-2-(pyrrolidine-1-carboxamido)propanamido)propanoate (18a). Compound **18a** was obtained by the general procedure D from **13a** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 83%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.04 (d, *J* = 8.5 Hz, 2H), 6.96 (brs, 1H), 6.78 (d, *J* = 8.5 Hz, 2H), 4.78 (m, 2H, CH + NH), 4.46–4.39 (m, 1H), 3.76 (s, 3H), 3.71 (s, 3H), 3.31 (m, 4H), 3.09 (dd, *J* = 14.0, 6.0 Hz, 1H), 3.00 (dd, *J* = 14.0, 6.5 Hz, 1H), 1.91 (m, 4H), 1.33 (d, *J* = 7.0 Hz, 3H); ESI-MS: *m/z* = 378 $[M + H]^+$.

5.1.8.6. Methyl(S)-3-(4-methoxyphenyl)-2-((R)-2-(pyrrolidine-1-carboxamido)propanamido)propanoate (18b). Compound **18b** was obtained by the general procedure D from **13b** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 85%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.04 (d, *J* = 8.5 Hz, 2H), 6.98 (d, *J* = 7.5 Hz, 1H), 6.80 (d, *J* = 8.5 Hz, 2H), 4.77 (m, 2H, CH + NH), 4.47–4.39 (m, 1H), 3.77 (s, 3H), 3.70 (s, 3H), 3.32 (m, 4H), 3.09 (dd, *J* = 14.0, 6.0 Hz, 1H), 3.01 (dd, *J* = 14.0, 6.5 Hz, 1H), 1.90 (m, 4H), 1.30 (d, *J* = 7.0 Hz, 3H). ESI-MS: *m/z* = 378 $[M + H]^+$.

5.1.8.7. Methyl(S)-3-(4-methoxyphenyl)-2-((S)-2-(1,2,3,4-tetrahydroisoquinoline-2-carboxamido)propanamido)propanoate (18c). Compound **18c** was obtained by the general procedure D from **13c** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 81%; Colorless oil; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.23–7.09 (m, 4H), 7.01 (d, *J* = 8.6 Hz, 2H), 6.79 (d, *J* = 7.4 Hz, 1H), 6.74 (d, *J* = 8.6 Hz, 2H), 5.03 (d, *J* = 7.0 Hz, 1H), 4.79 (m, 1H), 4.53 (s, 2H), 4.45 (m, 1H), 3.72 (s, 3H), 3.69 (s, 3H), 3.66–3.52 (m, 2H), 3.09 (dd, *J* = 14.0, 5.6 Hz, 1H), 3.00 (dd, *J* = 14.0,

6.4 Hz, 1H), 2.87 (t, $J = 6.0$ Hz, 2H), 1.36 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 440$ $[M + H]^+$.

5.1.8.8. Methyl(S)-3-(4-methoxyphenyl)-2-((R)-2-(1,2,3,4-tetrahydroisoquinoline-2-carboxamido)propanamido)propanoate (18d). Compound **18d** was obtained by the general procedure D from **13d** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 83%; Colorless oil; ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.20–7.09 (m, 4H), 7.03 (d, $J = 8.6$ Hz, 2H), 6.93 (d, $J = 7.8$ Hz, 1H), 6.77 (d, $J = 8.6$ Hz, 2H), 5.15 (d, $J = 7.0$ Hz, 1H), 4.80 (m, 1H), 4.53 (s, 2H), 4.46 (m, 1H), 3.72 (s, 3H), 3.69 (s, 3H), 3.66–3.52 (m, 2H), 3.10 (dd, $J = 14.0$, 5.6 Hz, 1H), 3.02 (dd, $J = 14.0$, 6.4 Hz, 1H), 2.86 (t, $J = 6.0$ Hz, 2H), 1.32 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 440$ $[M + H]^+$.

5.1.8.9. Methyl(S)-2-((S)-2-(9H-carbazole-9-carboxamido)propanamido)-3-(4-methoxyphenyl)propanoate (18e). Compound **18e** was obtained by the general procedure D from **17a** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 82%; White solid; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.06 (d, $J = 8.5$ Hz, 2H), 8.04 (d, $J = 7.5$ Hz, 2H), 7.52–7.46 (m, 2H), 7.39–7.33 (m, 2H), 7.01 (d, $J = 8.5$ Hz, 2H), 6.67 (d, $J = 8.5$ Hz, 2H), 6.49–6.43 (m, 2H, NH), 4.89 (m, 1H), 4.71 (m, 1H), 3.77 (s, 3H), 3.59 (s, 3H), 3.15 (dd, $J = 14.0$, 5.5 Hz, 1H), 3.03 (dd, $J = 14.0$, 6.5 Hz, 1H), 1.57 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 474$ $[M + H]^+$.

5.1.8.10. Methyl(S)-2-((R)-2-(9H-carbazole-9-carboxamido)propanamido)-3-(4-methoxyphenyl)propanoate (18f). Compound **18f** was obtained by the general procedure D from **17b** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 80%; White solid; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.05 (d, $J = 8.5$ Hz, 2H), 8.02 (d, $J = 7.5$ Hz, 2H), 7.48 (ddd, $J = 8.5$, 7.5, 1.0 Hz, 2H), 7.37–7.31 (m, 2H), 7.02 (d, $J = 8.5$ Hz, 2H), 6.77 (d, $J = 8.5$ Hz, 2H), 6.54 (d, $J = 7.0$ Hz, 1H), 6.49 (d, $J = 8.0$ Hz, 1H), 3.72 (s, 3H), 3.71 (s, 3H), 3.16 (dd, $J = 14.0$, 6.0 Hz, 1H), 3.09 (dd, $J = 14.1$, 6.0 Hz, 1H), 1.53 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 474$ $[M + H]^+$.

5.1.8.11. Methyl(2S)-3-(4-methoxyphenyl)-2-((2S)-2-(3-methoxypyrrolidine-1-carboxamido)propanamido)propanoate (18g). Compound **18g** was obtained by the general procedure D from **13g** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 83%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.03 (d, $J = 8.5$ Hz, 2H), 6.97 (m, 1H), 6.82–6.77 (m, 2H), 4.76 (m, 2H), 4.41 (m, 1H), 3.97 (m, 1H), 3.76 (s, 3H), 3.70 (s, 3H), 3.50–3.36 (m, 4H), 3.33 (m, 3H), 3.08 (dd, $J = 14.0$, 5.5 Hz, 1H), 3.04–2.95 (m, 1H), 2.08 (m, 1H), 1.97 (m, 1H), 1.32 (m, 3H); ESI-MS: $m/z = 408$ $[M + H]^+$.

5.1.8.12. Methyl(2S)-3-(4-methoxyphenyl)-2-((2R)-2-(3-methoxypyrrolidine-1-carboxamido)propanamido)propanoate (18h). Compound **18h** was obtained by the general procedure D from **13h** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 86%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.03 (d, $J = 8.5$ Hz, 2H), 6.90 (d, $J = 7.5$ Hz, 1H), 6.83–6.78 (m, 2H), 4.78 (m, 2H), 4.45–4.38 (m, 1H), 3.96 (m, 1H), 3.77 (s, 3H), 3.72–3.68 (m, 3H), 3.51–3.36 (m, 4H), 3.31 (d, 3H), 3.08 (m, 1H), 3.01 (m, 1H), 2.06 (m, 1H), 2.01–1.93 (m, 1H), 1.30 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 408$ $[M + H]^+$.

5.1.8.13. Methyl(S)-3-(4-methoxyphenyl)-2-((R)-2-(4-methoxypiperidine-1-carboxamido)propanamido)propanoate (18j). Compound **18j** was obtained by the general procedure D from **13j** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 83%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.02 (d, $J = 8.5$ Hz, 2H), 6.85 (d, $J = 7.5$ Hz, 1H), 6.80 (d, $J = 8.5$ Hz, 2H), 5.09 (d, $J = 6.5$ Hz, 1H), 4.78 (m, 1H), 4.39 (m, 1H), 3.77 (s, 3H), 3.70 (s, 3H), 3.66–3.58 (m, 2H), 3.37 (m, 1H), 3.34 (s, 3H), 3.15–3.05 (m, 3H), 3.00 (dd, $J = 14.0$, 6.5 Hz, 1H), 1.83 (m, 2H), 1.54 (m, 2H), 1.28 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 422$ $[M + H]^+$.

5.1.8.14. Methyl(S)-3-(4-methoxyphenyl)-2-((R)-2-((R)-3-methoxypyrrolidine-1-carboxamido)propanamido)propanoate (18k). Compound **18k** was obtained by the general procedure D from **13k** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 83%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.52 (brs, 1H), 7.07 (d, $J = 8.5$ Hz, 2H), 6.81 (d, $J = 8.5$ Hz, 2H), 5.55 (brs, 1H), 4.78–4.72 (m, 1H), 4.53–4.45 (m, 1H), 4.00–3.96 (m, 1H), 3.77 (s, 3H), 3.71 (s, 3H), 3.52–3.42 (m, 4H), 3.32 (s, 3H), 3.11 (dd, $J = 14.0$, 5.5 Hz, 1H), 3.04 (dd, $J = 14.0$, 7.0 Hz, 1H), 2.12–2.05 (m, 1H), 2.01–1.92 (m, 1H), 1.32 (d, $J = 7.0$ Hz, 3H). ESI-MS: $m/z = 408$ $[M + H]^+$.

5.1.8.15. Methyl(S)-3-(4-methoxyphenyl)-2-((R)-2-((S)-3-methoxypyrrolidine-1-carboxamido)propanamido)propanoate (18l). Compound **18l** was obtained by the general procedure D from **13l** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 79%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.04 (d, $J = 8.5$ Hz, 2H), 6.80 (d, $J = 8.5$ Hz, 2H), 4.91 (brs, 1H), 4.81–4.74 (m, 1H), 4.47–3.38 (s, 1H), 3.96 (m, 1H), 3.77 (s, 3H), 3.71 (s, 3H), 3.50 (m, 1H), 3.45–3.38 (m, 3H), 3.32 (s, 3H), 3.09 (dd, $J = 14.0$, 5.5 Hz, 1H), 3.02 (dd, $J = 14.0$, 6.5 Hz, 1H), 2.11–2.04 (m, 1H), 1.98 (m, 1H), 1.31 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 408$ $[M + H]^+$.

5.1.8.16. Methyl(S)-3-(4-methoxyphenyl)-2-((R)-2-((R)-3-methoxypiperidine-1-carboxamido)propanamido)propanoate (18 m). Compound **18m** was obtained by the general procedure D from **13m** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 80%; Colorless oil; ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.04 (d, $J = 8.5$ Hz, 2H), 6.81 (d, $J = 8.5$ Hz, 2H), 4.78 (dd, $J = 13.5$, 6.5 Hz, 1H), 4.42 (dd, $J = 13.0$, 6.0 Hz, 1H), 3.77 (s, 3H), 3.73–3.67 (m, 4H), 3.45–3.39 (m, 1H), 3.36 (s, 3H), 3.28–3.23 (m, 1H), 3.22–3.14 (m, 2H), 3.10 (dd, $J = 14.0$, 5.5 Hz, 1H), 3.02 (dd, $J = 14.0$, 6.5 Hz, 1H), 1.93–1.86 (m, 1H), 1.76 (m, 1H), 1.59–1.51 (m, 1H), 1.49–1.40 (m, 1H), 1.31 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 422$ $[M + H]^+$.

5.1.8.17. Methyl(S)-3-(4-methoxyphenyl)-2-((R)-2-((S)-3- methoxypiperidine-1-carboxamido)propanamido)propanoate (18n). Compound **18n** was obtained by the general procedure D from **13n** and methyl (*S*)-2-amino-3-(4-methoxyphenyl)propanoate hydrochloride. Yield: 88%; Colorless oil (153 mg, 86%); ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.02 (d, $J = 8.5$ Hz, 2H), 6.81 (d, $J = 8.5$ Hz, 3H), 5.09 (brs, 1H), 4.79 (m, 1H), 4.82–4.75 (m, 1H), 3.77 (s, 3H), 3.74–3.68 (m, 4H), 3.45–3.39 (m, 1H), 3.35 (s, 3H), 3.27–3.21 (m, 1H), 3.19–3.12 (m, 1H), 3.11–2.99 (m, 3H), 1.91 (m, 1H), 1.80–1.71 (m, 1H), 1.58–1.49 (m, 1H), 1.47–1.41 (m, 1H), 1.30 (d, $J = 7.0$ Hz, 3H); ESI-MS: $m/z = 422$ $[M + H]^+$.

5.1.8.18. N-(1-(((S)-1-(((S)-3-(1H-indol-3-yl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamoyl)piperidin-4-yl) thiazole-2-carboxamide (11a). Compound **11a** was obtained by the general procedure D from **7a** and **10a.** Yield:53%; white soild; mp:153–154 °C;^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.64 (s, 1H), 8.97 (s, 1H), 7.51 (d, $J = 8.0$ Hz, 1H), 7.45 (d, $J = 3.5$ Hz, 1H), 7.29 (d, $J = 8.0$ Hz, 2H), 7.18–7.13 (m, 3H), 7.11 (t, $J = 7.3$ Hz, 2H), 7.04 (d, $J = 7.5$ Hz, 1H), 7.01 (d, $J = 3.5$ Hz, 1H), 6.96–6.92 (m, 2H), 6.90 (s, 1H), 5.30 (s, 1H), 4.78–4.67 (m, 2H), 4.37–4.29 (m, 1H), 3.87 (d, $J = 12.5$ Hz, 1H), 3.75 (d, $J = 12.5$ Hz, 1H), 3.24 (dd, $J = 15.0$, 5.5 Hz,

1H), 3.18 (d, J = 5.0 Hz, 1H), 3.09 (dd, J = 15.0, 6.0 Hz, 1H), 2.93 (dd, J = 14.0, 5.5 Hz, 1H), 2.76 (d, J = 5.0 Hz, 1H), 2.69 (dd, J = 23.8, 11.8 Hz, 2H), 2.61–2.53 (m, 2H), 1.80–1.67 (m, 2H), 1.66–1.54 (m, 2H), 1.37 (s, 3H), 1.27 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.55, 173.72, 172.92, 171.26, 159.29, 156.81, 136.70, 136.21, 135.88, 129.23, 128.40, 127.36, 126.88, 123.23, 122.05, 119.43, 118.60, 114.02, 111.42, 109.80, 59.14, 53.35, 52.37, 50.63, 43.30, 43.07, 42.16, 36.93, 27.82, 27.30, 18.24, 16.39; ESI-MS: m/z = 700 $[M+H]^+$.

5.1.8.19. N-(1-(((R)-1-(((S)-3-(1H-indol-3-yl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamoyl)piperidin-4-yl) thiazole-2-carboxamide (11b). Compound **11b** was obtained by the general procedure D from **7b** and **10a.** Yield:53%; white soild; mp:153–154 °C;^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.66 (s, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.44 (d, J = 4.0 Hz, 1H), 7.39 (d, J = 7.5 Hz, 1H), 7.33 (d, J = 8.0 Hz, 1H), 7.22–7.16 (m, 3H), 7.13 (t, J = 7.5 Hz, 1H), 7.08–7.02 (m, 3H), 7.01–6.95 (m, 2H), 6.81 (s, 1H), 5.92 (s, 1H), 4.74–4.68 (m, 2H), 4.12–4.05 (m, 1H), 4.01–3.87 (m, 2H), 3.29–3.23 (m, 2H), 3.11 (dd, J = 15.0, 6.0 Hz, 1H), 2.96 (dd, J = 13.5, 4.5 Hz, 1H), 2.87–2.65 (m, 5H), 1.91 (d, J = 11.5 Hz, 1H), 1.84 (d, J = 11.0 Hz, 1H), 1.70–1.59 (m, 2H), 1.39 (s, 3H), 1.28 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.72, 174.64, 173.16, 171.54, 160.08, 157.35, 136.55, 136.09, 133.42, 129.43, 128.41, 127.42, 126.69, 123.22, 122.05, 119.49, 118.43, 113.82, 111.39, 109.64, 59.23, 53.56, 53.19, 52.47, 51.34, 43.29, 43.09, 42.10, 36.49, 27.95, 27.60, 27.04, 17.57, 16.50; ESI-MS: m/z = 700 $[M+H]^+$.

5.1.8.20. N-(1-(((S)-1-(((S)-1-(((S)-3-cyclohexyl-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)amino)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamoyl)piperidin-4-yl) thiazole-2-carboxamide (11c). Compound **11c** was obtained by the general procedure D from **7a** and **10b.** Yield:65%; white soild; mp:162–163 °C;^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.60 (s, 1H), 9.14 (s, 1H), 7.50 (d, J = 8.0 Hz, 1H), 7.48 (d, J = 3.5 Hz, 1H), 7.42 (s, 1H), 7.30 (d, J = 8.0 Hz, 1H), 7.16 (s, 1H), 7.10 (t, J = 7.5 Hz, 1H), 7.04–6.98 (m, 3H), 5.20 (s, 1H), 4.80–4.72 (m, 1H), 4.57–4.49 (m, 1H), 4.40–4.32 (m, 1H), 3.90 (d, J = 12.5 Hz, 1H), 3.74 (d, J = 12.5 Hz, 1H), 3.28 (dd, J = 15.0, 6.0 Hz, 1H), 3.24 (d, J = 5.0 Hz, 1H), 3.16 (dd, J = 15.0, 6.0 Hz, 1H), 2.79 (d, J = 5.0 Hz, 1H), 2.76–2.60 (m, 3H), 1.82–1.52 (m, 9H), 1.45 (s, 5H), 1.34 (d, J = 7.0 Hz, 3H), 1.17–1.01 (m, 5H), 0.87–0.75 (m, 2H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 208.48, 173.69, 172.96, 171.65, 159.23, 156.75, 136.74, 136.28, 127.38, 123.09, 122.04, 119.41, 118.59, 114.03, 111.39, 109.94, 59.06, 53.33, 52.40, 50.63, 49.60, 43.35, 43.04, 42.21, 38.17, 34.13, 33.75, 31.83, 27.83, 27.18, 26.27, 26.10, 25.89, 18.30, 16.74; ESI-MS: m/z = 706 $[M+H]^+$.

5.1.8.21. N-(1-(((R)-1-(((S)-1-(((S)-3-cyclohexyl-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)amino)-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamoyl)piperidin-4-yl) thiazole-2-carboxamide (11d). Compound **11d** was obtained by the general procedure D from **7b** and **10b.** Yield:60%; white soild; mp:190–191 °C;^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.92 (s, 1H), 7.54 (d, J = 8.0 Hz, 1H), 7.40 (d, J = 3.0 Hz, 1H), 7.37 (d, J = 7.0 Hz, 1H), 7.35 (d, J = 8.0 Hz, 1H), 7.17 (d, J = 6.0 Hz, 1H), 7.13 (t, J = 7.5 Hz, 1H), 7.09 (s, 1H), 7.04 (t, J = 7.5 Hz, 1H), 6.98 (d, J = 3.0 Hz, 1H), 5.88 (s, 1H), 4.81–4.73 (m, 1H), 4.55–4.48 (m, 1H), 4.19–4.11 (m, 1H), 3.96 (d, J = 12.0 Hz, 1H), 3.90 (d, J = 12.0 Hz, 1H), 3.36–3.18 (m, 3H), 2.82–2.72 (m, 3H), 2.71–2.61 (m, 1H), 1.88–1.76 (m, 2H), 1.75–1.60 (m, 5H), 1.60–1.53 (m, 2H), 1.46–1.41 (m, 5H), 1.29–1.15 (m, 6H), 1.12–1.01 (m, 2H), 0.88–0.72 (m, 2H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 208.77, 174.52, 173.02, 171.77, 159.61, 157.31, 136.26, 135.56, 127.43, 123.39, 122.04, 119.50, 118.51, 113.85, 111.40, 110.02, 59.11, 53.64, 52.51, 52.4, 51.20, 49.84, 49.56, 43.24, 42.14, 37.85, 34.15, 33.74, 31.95, 31.84, 27.88, 27.79, 27.34, 26.30, 26.21, 26.09, 25.89, 17.83, 16.76; ESI-MS: m/z = 706 $[M+H]^+$.

5.1.8.22. N-(1-(((S)-1-(((S)-1-(((S)-3-cyclohexyl-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)amino)-3-(4-methoxyphenyl)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamoyl)piperidin-4-yl)thiazole-2-carboxamide (11e). Compound **11e** was obtained by the general procedure D from **7a** and **10c.** Yield:65%; white soild; mp:108–110 °C;^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.64 (s, 1H), 7.56–7.42 (m, 2H), 7.18–7.07 (m, 1H), 7.02 (d, J = 3.5 Hz, 1H), 6.98 (d, J = 8.0 Hz, 2H), 6.73 (d, J = 8.0 Hz, 2H), 5.29 (s, 1H), 4.68–4.63 (m, 1H), 4.63–4.55 (m, 1H), 4.52–4.42 (m, 1H), 4.01 (d, J = 13.0 Hz, 1H), 3.93 (d, J = 13.0 Hz, 1H), 3.74 (s, 3H), 3.29 (d, J = 5.0 Hz, 1H), 2.98–2.86 (m, 4H), 2.85–2.77 (m, 1H), 2.72–2.65 (m, 1H), 1.93–1.72 (m, 5H), 1.70–1.60 (m, 3H), 1.55–1.45 (m, 5H), 1.34 (d, J = 7.0 Hz, 3H), 1.24–1.10 (m, 5H), 0.95–0.81 (m, 2H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 208.46, 173.87, 172.68, 171.06, 159.42, 158.45, 156.96, 136.72, 130.24, 128.41, 113.85, 113.83, 59.01, 55.20, 54.49, 52.34, 50.14, 49.32, 43.34, 43.15, 42.05, 38.37, 36.64, 34.24, 33.82, 31.85, 27.81, 27.76, 26.29, 26.17, 25.92, 18.68, 16.68; ESI-MS: m/z = 697 $[M+H]^+$.

5.1.8.23. N-(1-(((R)-1-(((S)-1-(((S)-3-cyclohexyl-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)amino)-3-(4-methoxyphenyl)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)carbamoyl)piperidin-4-yl)thiazole-2-carboxamide (11f). Compound **11f** was obtained by the general procedure D from **7b** and **10c.** Yield:65%; white soild; mp: 134–136 °C;^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.77 (s, 1H), 7.38 (d, J = 3.0 Hz, 1H), 7.08 (d, J = 8.5 Hz, 2H), 6.98 (d, J = 3.5 Hz, 1H), 6.88 (d, J = 8.0 Hz, 1H), 6.81 (d, J = 8.5 Hz, 2H), 5.78 (s, 1H), 4.71–4.62 (m, 1H), 4.60–4.53 (m, 1H), 4.21–4.13 (m, 1H), 4.00 (d, J = 12.5 Hz, 1H), 3.93 (d, J = 12.5 Hz, 1H), 3.77 (s, 3H), 3.34 (d, J = 5.0 Hz, 1H), 3.12–2.97 (m, 2H), 2.93–2.80 (m, 3H), 2.74–2.63 (m, 1H), 1.93–1.82 (m, 2H), 1.80–1.65 (m, 4H), 1.64–1.50 (m, 4H), 1.45 (s, 3H), 1.34–1.20 (m, 5H), 1.16–1.04 (m, 3H), 0.95–0.80 (m, 2H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 208.64, 174.29, 172.82, 171.20, 159.57, 158.61, 157.55, 136.41, 131.30, 130.37, 128.30, 114.04, 59.13, 55.24, 54.08, 52.43, 51.00, 49.67, 43.32, 43.28, 42.23, 37.91, 36.64, 34.20, 33.88, 31.86, 27.87, 26.35, 26.17, 25.91, 17.98, 16.76; ESI-MS: m/z = 697 $[M+H]^+$.

5.1.8.24. N-((S)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)pyrrolidine-1-carboxamide (20a). Compound **20a** was obtained by the general procedure D from **8a** and **19a.** Yield: 68%; White solid; mp = 74–75 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.26–7.20 (m, 3H), 7.08 (d, J = 7.0 Hz, 2H), 7.04 (d, J = 8.5 Hz, 3H), 6.78 (d, J = 7.0 Hz, 1H), 6.74 (d, J = 8.5 Hz, 2H), 4.81–4.73 (m, 1H), 4.59–4.45 (m, 2H), 4.32–4.24 (m, 1H), 3.75 (s, 3H), 3.30 (d, J = 5.0 Hz, 1H), 3.27–3.17 (m, 4H), 3.05 (dd, J = 14.0, 5.0 Hz, 1H), 2.95 (d, J = 7.0 Hz, 2H), 2.87 (d, J = 5.0 Hz, 1H), 2.74 (dd, J = 14.0, 8.0 Hz, 1H), 1.95–1.82 (m, 4H), 1.45 (s, 3H), 1.28 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.20, 173.35, 170.79, 158.45, 156.19, 135.97, 130.28, 129.30, 128.65, 128.46, 126.97, 113.84, 59.19, 55.20, 54.13, 52.42, 49.92, 45.63, 37.04, 36.37, 25.46, 18.13, 16.46; ESI-MS: m/z = 551 $[M + H]^+$.

5.1.8.25. N-((R)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)pyrrolidine-1-carboxamide (20b). Compound **19b** was obtained by the general procedure D from **8a** and **18b.** Yield: 65%; White solid; mp = 181–182 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.25–7.17

(m, 3H), 7.12–7.08 (m, 2H), 7.03 (m, 3H), 6.84 (d, J = 8.5 Hz, 1H), 6.75 (d, J = 8.5 Hz, 2H), 4.78–4.71 (m, 1H), 4.66 (d, J = 7.0 Hz, 1H), 4.58–4.51 (m, 1H), 4.21–4.13 (m, 1H), 3.76 (s, 3H), 3.38–3.20 (m, 5H), 3.02 (dd, J = 13.5, 5.0 Hz, 1H), 2.98–2.87 (m, 2H), 2.85 (d, J = 5.0 Hz, 1H), 2.76 (dd, J = 14.0, 8.5 Hz, 1H), 1.89 (s, 4H), 1.42 (s, 3H), 1.23 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.37, 173.47, 170.90, 158.51, 156.49, 136.32, 130.31, 129.38, 128.57, 128.41, 126.87, 113.98, 59.19, 55.23, 54.13, 52.73, 52.42, 50.29, 45.67, 36.91, 36.47, 25.49, 17.97, 16.46; ESI-MS: m/z = 551 $[M + H]^+$.

5.1.8.26. N-((S)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)-3,4-dihydroisoquinoline-2(1H)-carboxamide (20c). Compound **20c** was obtained by the general procedure D from **8a** and **19c.** Yield: 63%; White solid; mp = 178–179 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.26–7.10 (m, 7H), 7.08–6.99 (m, 4H), 6.91 (d, J = 7.5 Hz, 1H), 6.69 (d, J = 8.5 Hz, 2H), 6.59 (d, J = 7.5 Hz, 1H), 4.89 (d, J = 5.0 Hz, 1H), 4.82–4.73 (m, 1H), 4.54–4.46 (m, 3H), 4.38–4.27 (m, 1H), 3.64 (s, 3H), 3.60–3.50 (m, 2H), 3.28 (d, J = 5.0 Hz, 1H), 3.06 (dd, J = 14.0, 5.0 Hz, 1H), 2.94 (d, J = 6.5 Hz, 2H), 2.90–2.81 (m, 3H), 2.72 (dd, J = 14.0, 8.0 Hz, 1H), 1.46 (s, 3H), 1.30 (d, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 207.07, 173.18, 170.60, 158.53, 156.97, 135.79, 134.91, 132.99, 130.26, 129.26, 128.51, 128.47, 128.34, 127.00, 126.83, 126.54, 126.34, 113.92, 59.15, 55.09, 54.23, 52.41, 50.11, 45.40, 41.24, 37.11, 36.57, 28.97, 18.24, 16.44; ESI-MS: m/z = 613 $[M + H]^+$.

5.1.8.27. (S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl(S)-3-(4-methoxyphenyl)-2-((R)-2-(1,2,3,4-tetrahydroisoquinoline-2-carboxamido)propanamido)propanoate (20d). Compound **20d** was obtained by the general procedure D from **8a** and **19d.** Yield: 60%; White solid; mp = 76–77 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.25–7.13 (m, 6H), 7.12–7.00 (m, 5H), 6.95 (d, J = 7.5 Hz, 1H), 6.86 (d, J = 8.0 Hz, 1H), 6.75 (d, J = 8.5 Hz, 2H), 5.18 (d, J = 7.0 Hz, 1H), 4.81–4.71 (m, 1H), 4.63–4.48 (m, 3H), 4.30–4.18 (m, 1H), 3.74 (s, 3H), 3.68–3.60 (m, 1H), 3.58–3.52 (m, 1H), 3.28 (d, J = 5.0 Hz, 1H), 3.01 (dd, J = 14.0, 5.0 Hz, 1H), 2.97–2.89 (m, 2H), 2.87–2.79 (m, 3H), 2.73 (dd, J = 14.0, 8.5 Hz, 1H), 1.38 (s, 3H), 1.25 (d, J = 7.0 Hz, 4H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 207.31, 173.50, 170.77, 158.56, 157.27, 136.11, 134.96, 133.14, 130.29, 129.33, 128.46, 128.41, 128.35, 126.89, 126.72, 126.43, 126.33, 114.01, 59.12, 55.19, 54.14, 52.59, 52.36, 50.50, 45.48, 41.28, 37.07, 36.56, 28.95, 18.17, 16.37; ESI-MS: m/z = 613 $[M + H]^+$.

5.1.8.28. N-((S)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)-9H-carbazole-9-carboxamide (20e). Compound **20e** was obtained by the general procedure D from **8a** and **19e.** Yield: 61%; White solid; mp = 187–188 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.04–7.99 (m, 4H), 7.49–7.43 (m, 2H), 7.34 (t, J = 7.5 Hz, 2H), 7.19–7.13 (m, 3H), 7.08 (d, J = 8.5 Hz, 2H), 6.94 (dd, J = 7.0, 2.0 Hz, 2H), 6.88 (d, J = 7.5 Hz, 1H), 6.64–6.56 (m, 3H), 6.37 (d, J = 7.5 Hz, 1H), 4.76–4.69 (m, 1H), 4.68–4.62 (m, 2H), 3.52 (s, 3H), 3.18 (d, J = 5.0 Hz, 1H), 3.02–2.93 (m, 3H), 2.85 (d, J = 5.0 Hz, 1H), 2.61 (dd, J = 14.0, 8.0 Hz, 1H), 1.48 (d, J = 7.0 Hz, 3H), 1.44 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 206.99, 171.94, 170.28, 158.61, 152.34, 138.20, 135.43, 130.36, 129.21, 128.53, 127.99, 127.12, 125.25, 122.56, 120.14, 113.96,113.89, 59.18, 54.97, 54.43, 52.66, 52.41, 50.08, 37.51, 37.04, 18.86, 16.46, 0.02; ESI-MS: m/z = 647 $[M + H]^+$.

5.1.8.29. N-((R)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)-9H-carbazole-9-carboxamide (20f). Compound **20f** was obtained by the general procedure D from **8a** and **19f.** Yield: 62%; White solid; mp = 120–121 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.06 (d, J = 8.5 Hz, 2H), 8.02 (t, J = 7.5 Hz, 2H), 7.51–7.45 (m, 2H), 7.35 (t, J = 7.5 Hz, 2H), 7.12 (t, J = 7.5 Hz, 2H), 7.09–7.04 (m, 3H), 6.96 (d, J = 7.0 Hz, 2H), 6.77–6.70 (m, 3H), 6.61 (d, J = 7.0 Hz, 1H), 6.32 (d, J = 7.0 Hz, 1H), 4.72–4.66 (m, 1H), 4.66–4.62 (m, 1H), 4.62–4.57 (m, 1H), 3.72 (s, 3H), 3.21 (d, J = 5.0 Hz, 1H), 3.04–2.96 (m, 2H), 2.91 (dd, J = 14.0, 7.0 Hz, 1H), 2.85 (d, J = 5.0 Hz, 1H), 2.59 (dd, J = 14.0, 8.5 Hz, 1H), 1.45 (d, J = 7.0 Hz, 3H), 1.41 (s, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 206.92, 171.96, 170.25, 158.73, 152.33, 138.25, 135.47, 130.41, 129.19, 128.49, 128.02, 127.13, 127.08, 125.27, 122.54, 120.14, 114.07, 113.91, 59.22, 55.22, 54.25, 52.83, 52.44, 50.30, 37.03, 36.98, 19.11, 16.45; ESI-MS: m/z = 647 $[M + H]^+$.

5.1.8.30. 3-Methoxy-N-((S)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)pyrrolidine-1-carboxamide (20g). Compound **20g** was obtained by the general procedure D from **8a** and **19g.** Yield: 63%; White solid; mp = 66–67 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.25–7.18 (m, 3H), 7.08–7.02 (m, 4H), 7.01–6.92 (m, 1H), 6.79–6.71 (m, 2H), 6.70–6.61 (m, 1H), 4.79–4.72 (m, 1H), 4.57 (s, 1H), 4.52–4.45 (m, 1H), 4.29–4.21 (m, 1H), 3.98–3.92 (m, 1H), 3.75 (s, 3H), 3.47–3.34 (m, 3H), 3.33–3.30 (m, 4H), 3.29–3.27 (m, 1H), 3.08–3.01 (m, 1H), 2.99–2.85 (m, 3H), 2.76–2.67 (m, 1H), 2.10–2.03 (m, 1H), 2.01–1.90 (m, 1H), 1.45 (s, 3H), 1.26 (m, 3H); ESI-MS: m/z = 581 $[M + H]^+$.

5.1.8.31. 3-Methoxy-N-((R)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)pyrrolidine-1-carboxamide (20h). Compound **20h** was obtained by the general procedure D from **8a** and **19h.** Yield: 70%; White solid; mp = 167–168 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.26–7.18 (m, 3H), 7.10 (d, J = 7.0 Hz, 2H), 7.04 (d, J = 8.0 Hz, 2H), 6.94 (d, J = 7.5 Hz, 1H), 6.81–46.73 (m, 3H), 4.80–4.73 (m, 1H), 4.71–4.63 (m, 1H), 4.58–4.50 (m, 1H), 4.23–4.14 (m, 1H), 4.01–3.91 (m, 1H), 3.76 (s, 3H), 3.55–3.35 (m, 4H), 3.34–3.29 (m, 4H), 3.02 (dd, J = 14.0, 5.0 Hz, 1H), 2.95–2.90 (m, 2H), 2.86 (d, J = 5.0 Hz, 1H), 2.76 (dd, J = 13.5, 8.5 Hz, 1H), 2.11–1.92 (m, 2H), 1.43 (s, 3H), 1.26–1.18 (m, 3H); ESI-MS: m/z = 581 $[M + H]^+$.

5.1.8.32. 4-Methoxy-N-((S)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)piperidine-1-carboxamide (20i). Compound **20i** was obtained by the general procedure D from **8a** and **19i.** Yield: 65%; White solid; mp = 76–78 °C. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.24–7.15 (m, 3H), 7.09–6.94 (m, 5H), 6.79 (d, J = 7.6 Hz, 1H), 6.73 (d, J = 8.0 Hz, 2H), 5.10 (d, J = 6.4 Hz, 1H), 4.81–4.70 (m, 1H), 4.57–4.44 (m, 1H), 4.43–4.20 (m, 1H), 3.73 (s, 3H), 3.63–3.50 (m, 2H), 3.40–3.29 (m, 4H), 3.25 (d, J = 5.0 Hz, 1H), 3.18–2.96 (m, 3H), 2.94–2.87 (m, 2H), 2.84 (d, J = 5.0 Hz, 1H), 2.70 (dd, J = 14.0, 8.0 Hz, 1H), 1.78 (s, 2H), 1.58–1.45 (m, 2H), 1.41 (s, 3H), 1.24 (d, J = 7.0 Hz, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 207.24, 173.40, 170.69, 158.49, 156.93, 135.85, 130.30, 129.25, 128.49, 128.44, 126.96, 113.90, 75.28, 59.11, 55.67, 55.16, 54.26, 52.33, 50.24, 41.14, 37.12, 36.70, 30.27, 18.47, 16.39; ESI-MS: m/z = 595 $[M + H]^+$.

5.1.8.33. 4-Methoxy-N-((R)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)piperidine-1-carboxamide (20j). Compound **20j** was obtained by the general procedure D from **8a** and **19j.** Yield: 73%; White solid; mp = 78–79 °C. 1H NMR (500 MHz, Chloroform-*d*) δ 7.25–7.18 (m, 3H), 7.12–7.09 (m, 2H),

7.02 (d, J = 8.5 Hz, 2H), 6.92 (d, J = 7.5 Hz, 1H), 6.78–6.66 (m, 3H), 5.10 (s, 1H), 4.78–4.71 (m, 1H), 4.58–4.48 (m, 1H), 4.14 (s, 1H), 3.76 (s, 3H), 3.69–3.62 (m, 1H), 3.61–3.54 (m, 1H), 3.41–3.35 (m, 1H), 3.34 (s, 3H), 3.31 (d, J = 5.0 Hz, 1H), 3.20–3.07 (m, 2H), 3.02 (dd, J = 13.5, 5.0 Hz, 1H), 2.93–2.89 (m, 2H), 2.86 (d, J = 5.0 Hz, 1H), 2.72 (dd, J = 13.5, 8.5 Hz, 1H), 1.87–1.80 (m, 2H), 1.59–1.50 (m, 2H), 1.42 (s, 3H), 1.22 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.31, 173.58, 170.81, 158.56, 157.28, 136.22, 130.29, 129.37, 128.46, 128.39, 126.92, 114.03, 75.33, 59.19, 55.70, 55.23, 54.11, 52.71, 52.42, 50.58, 41.28, 41.21, 36.98, 36.53, 30.27, 30.24, 18.01, 16.46; ESI-MS: m/z = 595 $[M + H]^+$.

5.1.8.34. (R)-3-Methoxy-N-((R)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl) amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)pyrrolidine-1-carboxamide (20k). Compound **20k** was obtained by the general procedure D from **8a** and **19k.** Yield: 69%; White solid; mp = 74–75 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.25–7.18 (m, 3H), 7.14–7.09 (m, 2H), 7.03 (d, J = 8.5 Hz, 2H), 6.96 (d, J = 7.0 Hz, 1H), 6.76 (d, J = 8.5 Hz, 3H), 4.79–4.72 (m, 1H), 4.71 (s, 1H), 4.56–4.49 (m, 1H), 4.20–4.13 (m, 1H), 3.97 (s, 1H), 3.77 (s, 3H), 3.48–3.38 (m, 4H), 3.35–3.30 (m, 4H), 3.02 (dd, J = 13.5, 5.0 Hz, 1H), 2.92 (d, J = 6.5 Hz, 2H), 2.87 (d, J = 5.0 Hz, 1H), 2.76 (dd, J = 14.0, 8.5 Hz, 1H), 2.09–2.01 (m, 1H), 1.99–1.93 (m, 1H), 1.42 (s, 3H), 1.24 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.46, 173.38, 170.83, 158.54, 156.51, 136.26, 130.31, 129.40, 128.48, 128.44, 126.90, 114.02, 79.54, 59.19, 56.58, 55.24, 54.17, 52.70, 52.45, 50.68, 50.35, 43.81, 36.93, 36.49, 30.75, 17.99, 16.46; ESI-MS: m/z = 581 $[M + H]^+$.

5.1.8.35. (S)-3-Methoxy-N-((R)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl) amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)pyrrolidine-1-carboxamide (20l). Compound **20l** was obtained by the general procedure D from **8a** and **19l.** Yield: 62%; White solid; mp = 185–186 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.26–7.17 (m, 3H), 7.12–7.09 (m, 2H), 7.04 (d, J = 8.5 Hz, 2H), 6.94 (d, J = 7.0 Hz, 1H), 6.81 (s, 1H), 6.76 (d, J = 8.5 Hz, 2H), 4.78–4.71 (m, 1H), 4.68 (d, J = 6.0 Hz, 1H), 4.58–4.50 (m, 1H), 4.22–4.13 (m, 1H), 3.94 (s, 1H), 3.76 (s, 3H), 3.55–3.45 (m, 1H), 3.44–3.34 (m, 3H), 3.33–3.28 (m, 4H), 3.02 (dd, J = 14.0, 5.0 Hz, 1H), 2.96–2.88 (m, 2H), 2.86 (d, J = 5.0 Hz, 1H), 2.76 (dd, J = 14.0, 8.5 Hz, 1H), 2.10–2.03 (m, 1H), 2.01–1.93 (m, 1H), 1.42 (s, 3H), 1.23 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.39, 173.35, 170.85, 158.53, 156.50, 136.23, 130.31, 129.39, 128.52, 128.44, 126.91, 114.00, 79.25, 59.18, 56.60, 55.24, 54.18, 52.71, 52.44, 50.63, 50.31, 43.61, 36.95, 36.52, 31.03, 17.97, 16.46; ESI-MS: m/z = 581 $[M + H]^+$.

5.1.8.36. (R)-3-Methoxy-N-((R)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl) amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)piperidine-1-carboxamide (20 m). Compound **20m** was obtained by the general procedure D from **8a** and **19m.** Yield: 60%; White solid; mp = 78–79 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.25–7.17 (m, 3H), 7.13 (d, J = 7.0 Hz, 2H), 7.04–6.96 (m, 3H), 6.75 (d, J = 8.5 Hz, 2H), 6.64 (d, J = 7.0 Hz, 1H), 5.06 (d, J = 5.5 Hz, 1H), 4.79–4.70 (m, 1H), 4.56–4.47 (m, 1H), 4.17–4.05 (m, 1H), 3.76 (s, 3H), 3.65 (d, J = 12.5 Hz, 1H), 3.46–3.39 (m, 1H), 3.36–3.29 (m, 4H), 3.29–3.23 (m, 1H), 3.19–3.09 (m, 2H), 3.02 (dd, J = 14.0, 5.0 Hz, 1H), 2.93 (d, J = 6.5 Hz, 2H), 2.85 (d, J = 5.0 Hz, 1H), 2.74 (dd, J = 14.0, 8.5 Hz, 1H), 1.94–1.87 (m, 1H), 1.79–1.70 (m, 1H), 1.58–1.51 (m, 1H), 1.50–1.43 (m, 1H), 1.41 (s, 3H), 1.22 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.36, 173.61, 170.84, 158.49, 157.52, 136.27, 130.27, 129.36, 128.51, 128.43, 126.90, 113.98, 74.55, 59.18, 56.21, 55.22, 54.14, 52.67, 52.39, 50.74, 47.70, 44.55, 36.95, 36.34, 29.25, 22.21, 17.95, 16.43; ESI-MS: m/z = 595 $[M + H]^+$.

5.1.8.37. (S)-3-Methoxy-N-((R)-1-(((S)-3-(4-methoxyphenyl)-1-(((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl) amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)piperidine-1-carboxamide (20n). Compound **20n** was obtained by the general procedure D from **8a** and **19n.** Yield: 64%; White solid; mp = 77–78 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.26–7.18 (m, 3H), 7.12 (d, J = 7.0 Hz, 2H), 7.03 (d, J = 8.5 Hz, 2H), 6.91 (d, J = 6.5 Hz, 1H), 6.76 (d, J = 8.5 Hz, 2H), 5.18 (s, 1H), 4.79–4.71 (m, 1H), 4.56–4.49 (m, 1H), 4.16 (s, 1H), 3.81–3.73 (m, 4H), 3.45–3.38 (m, 1H), 3.35 (s, 3H), 3.32 (d, J = 5.0 Hz, 1H), 3.29–3.23 (m, 1H), 3.22–3.16 (m, 1H), 3.06–2.97 (m, 2H), 2.92 (d, J = 6.5 Hz, 2H), 2.86 (d, J = 5.0 Hz, 1H), 2.75 (dd, J = 14.0, 8.5 Hz, 1H), 1.95–1.88 (m, 1H), 1.79–1.70 (m, 1H), 1.58–1.49 (m, 1H), 1.49–1.39 (m, 4H), 1.25 (d, J = 7.0 Hz, 3H); ^{13}C NMR (126 MHz, Chloroform-*d*) δ 207.32, 173.51, 170.80, 158.56, 157.54, 136.21, 130.31, 129.36, 128.47, 128.38, 126.94, 114.04, 74.62, 59.19, 56.24, 55.24, 54.07, 52.71, 52.42, 50.66, 48.01, 44.46, 36.98, 36.50, 29.43, 22.43, 17.90, 16.46; ESI-MS: m/z = 595 $[M + H]^+$.

5.2. In vitro assays of chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome and immunoproteasome

The human constitutive proteasome was given by Dr. Jiang-ping Wu (Notre-Dame Hospital, Montreal, QC, Canada), derived from human hepatic cells; the immunoproteasome was purchased from Boston Biochem, derived from human peripheral blood mononuclear cell. We used several fluorogenic substrates, the fluorogenic substrate Suc-LLE-AMC was used to measure the Capase-Like activity of the proteasome, β1c. Suc-KQL-AMC was used to measure the Trypsin-Like activity of the proteasome, β2c. The fluorogenic substrate Suc-PAL-AMC was used to measure the Capase-Like activity of the immunoproteasome, β1i. Suc-VGR-AMC was used to measure the Trypsin-Like activity of the immunoproteasome, β2i. Suc-WLA-AMC was used to measure the Chymotrypsin-Like activity of β5c and Suc-ANW-AMC was used to measure the Chymotrypsin-Like activity of β5i. All compounds were solved in DMSO-d_6, and then diluted with deionization water to be 10% DMSO-d_6 containing solution. Buffer conditions: 100 mM Tris-HCl, pH 7.5. In the assay, ONX-0914 was used as Positive control, and 2% DMSO-d_6 was used as negative control. The blank was no enzyme which was replaced by buffer. Briefly, first add 20 μL of the human proteasome (40 μg/mL) or immunoproteasome (1 μg/mL) per well. Subsequently add 10 μL of the sample. Cover with a adhesive strip and incubated 15 min at the at room temperature (25 °C). Then 20 μL of the flurogenic substrates (100 μM final) were added into the each well. Covered with a new adhesive strip and incubated 1 h at the room temperature (25 °C). Signal from cleaved AMC molecule was detected on EnVision® Multilabel Plate Reader with a 355 nm excitation filter and a 460 nm emission filter. IC_{50} data was calculated using GraphPad Prism software, and the equation'-sigmoidal dose–response (variable slope)' was chosen for curve fitting.

5.3. Cancer cell proliferation assay

5.3.1. Cell culture

RPMI-8226 and MM.1S cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The human MM cell lines PRMI 8226 and MM.1S were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin from Invitrogen (Grand Island, NY, USA).

5.3.2. Cell proliferation assays

A 90 μL aliquot of RPMI-8226 (5×10^3 cells per well) or MM.1S (3×10^4 cells per well) was seeded into 96-well plates and then treated with 10 μL of 0.2% DMSO-d_6 or varying concentrations of tested compounds for 72 h. Cell viability was measured using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS; Promega, Madison, WI). 20 μL of the combined MTS/PMS solution was pipetted into each of the 96-wells plate and then incubated for 2–4 h at 37 °C in a humidified, 5% CO_2 atmosphere. The optical density of each well was determined at 490 nm (background subtraction at 690 nm) using a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Growth inhibitory ratios were 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 a sigmoidal dose responsecurve (variable slope) and computed using GraphPad Prism version 5.02, GraphPad Software.

5.4. Molecular docking

The molecular docking was performed using Discovery Studio (version 2.1) with the default option. The X-ray crystal structure of immunoproteasome (PDB code: 5L5D) was used as the docking template. It was prepared by adding missing hydrogen bond, removing crystallographic waters and ligands with CHARMM force field. Three-dimensional (3D) conformation of compound **11a**, **11b, E-83, 20l** was prepared by Discovery Studio 2.1/Diverse conformation generation, then whose energy minimized by DS 2.1/Minimize molecule. For the docking calculation of compound **11a**, **11b, E-83, 20l** Discovery Studio 2.1/C-DOCKER was used to generate the conformations. The graphical image was analyzed by PyMOL v0.99.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations used

cCP	constitutive proteasome
CP	core particle
DCM	dichloromethane
DIPEA	*N*,*N*-diisopropylethylamine
DMSO-d_6	dimethyl sulfoxide
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
FBS	fetal bovine serum
FDA	Food and Drug Administration
HOBt	1-hydroxybenzotriazole
IC_{50}	half maximal inhibitory concentration
iCP	immunoproteasome
MHC-I	major histocompatibility complex class I
MM	multiple myeloma
RPs	regulatory particles
SAR	structure–activityrelationship
tCP	thymoproteasome
TFA	trifluoroacetic acid
THF	tetrahydrofuran.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113556.

References

[1] E.E. Manasanch, R.Z. Orlowski, Proteasome inhibitors in cancer therapy, Nature reviews, Clin. Oncol. 14 (2017) 417–433.
[2] J. Zhao, B. Zhai, S.P. Gygi, A.L. Goldberg, mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy, Proc. Natl. Acad. Sci. U.S.A. 112 (2015) 15790–15797.
[3] D. Voges, P. Zwickl, W. Baumeister, The 26S proteasome: a molecular machine designed for controlled proteolysis, Annu. Rev. Biochem. 68 (1999) 1015–1068.
[4] M. Groll, L. Ditzel, J. Löwe, D. Stock, M. Bochtler, H.D. Bartunik, R. Huber, Structure of 20S proteasome from yeast at 2.4 A resolution, Nature 386 (1997) 463–471.
[5] J. Löwe, D. Stock, B. Jap, P. Zwickl, W. Baumeister, R. Huber, Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution, Science (New York, N.Y.) 268 (1995) 533–539.
[6] P.M. Cromm, C.M. Crews, The proteasome in modern drug discovery: second life of a highly valuable drug target, ACS Cent. Sci. 3 (2017) 830–838.
[7] E.M. Huber, M. Basler, R. Schwab, W. Heinemeyer, C.J. Kirk, M. Groettrup, M. Groll, Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity, Cell 148 (2012) 727–738.
[8] D.A. Ferrington, D.S. Gregerson, Immunoproteasomes: structure, function, and antigen presentation, Progress in molecular biology and translational science 109 (2012) 75–112.
[9] M. Basler, C.J. Kirk, M. Groettrup, The immunoproteasome in antigen processing and other immunological functions, Curr. Opin. Immunol. 25 (2013) 74–80.
[10] T. Nitta, S. Murata, K. Sasaki, H. Fujii, A.M. Ripen, N. Ishimaru, S. Koyasu, K. Tanaka, Y. Takahama, Thymoproteasome shapes immunocompetent repertoire of CD8+ T cells, Immunity 32 (2010) 29–40.
[11] S. Murata, K. Sasaki, T. Kishimoto, S. Niwa, H. Hayashi, Y. Takahama, K. Tanaka, Regulation of CD8+ T cell development by thymus-specific proteasomes, Science (New York, N.Y.) 316 (2007) 1349–1353.
[12] R.C. Kane, A.T. Farrell, R. Sridhara, R. Pazdur, United States Food and Drug Administration approval summary: bortezomib for the treatment of progressive multiple myeloma after one prior therapy, Clin. Canc. Res. : Off. J. Am. Assoc. Canc. Res. 12 (2006) 2955–2960.
[13] M. Alsina, S. Trudel, R.R. Furman, P.J. Rosen, O.A. O'Connor, R.L. Comenzo, A. Wong, L.A. Kunkel, C.J. Molineaux, A. Goy, A phase I single-agent study of twice-weekly consecutive-day dosing of the proteasome inhibitor carfilzomib in patients with relapsed or refractory multiple myeloma or lymphoma, Clinical cancer research, Off. J. Am. Assoc. Canc. Res. 18 (2012) 4830–4840.
[14] P. Moreau, T. Masszi, N. Grzasko, N. Bahlis, M. Hansson, L. Pour, I. Sandhu, P. Ganly, B. Baker, S. Jackson, A.-M. Stoppa, D. Simpson, P. Gimsing, A. Palumbo, L. Garderet, M. Cavo, S. Kumar, C. Touzeau, F. Buadi, P. Richardson, Ixazomib, an investigational oral proteasome inhibitor (PI), in: Combination with Lenalidomide and Dexamethasone (IRd), Significantly Extends Progression-free Survival (PFS) for Patients (Pts) with Relapsed And/or Refractory Multiple Myeloma (RRMM), The Phase 3 Tourmaline-MM1 Study, 2015.
[15] R. Ettari, M. Zappalà, S. Grasso, C. Musolino, V. Innao, A. Allegra, Immunoproteasome-selective and non-selective inhibitors: a promising approach for the treatment of multiple myeloma, Pharmacol. Ther. 182 (2018) 176–192.
[16] D.J. Kuhn, R.Z. Orlowski, The immunoproteasome as a target in hematologic malignancies, Semin. Hematol. 49 (2012) 258–262.
[17] S. Downey-Kopyscinski, E.W. Daily, M. Gautier, A. Bhatt, B.I. Florea, C.S. Mitsiades, P.G. Richardson, C. Driessen, H.S. Overkleeft, A.F. Kisselev, An inhibitor of proteasome β2 sites sensitizes myeloma cells to immunoproteasome inhibitors, Blood advances 2 (2018) 2443–2451.
[18] D. Dytfeld, M. Luczak, T. Wrobel, L. Usnarska-Zubkiewicz, K. Brzezniakiewicz, K. Jamroziak, K. Giannopoulos, A. Przybylowicz-Chalecka, B. Ratajczak, J. Czerwinska-Rybak, Comparative proteomic profiling of refractory/relapsed multiple myeloma reveals biomarkers involved in resistance to bortezomib-based therapy, Oncotarget 7 (2016) 56726–56736.
[19] T. Muchamuel, M. Basler, M.A. Aujay, E. Suzuki, K.W. Kalim, C. Lauer, C. Sylvain, E.R. Ring, J. Shields, J. Jiang, P. Shwonek, F. Parlati, S.D. Demo,

M.K. Bennett, C.J. Kirk, M. Groettrup, A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis, Nat. Med. 15 (2009) 781–787.
[20] A.V. Singh, M. Bandi, M.A. Aujay, C.J. Kirk, D.E. Hark, N. Raje, D. Chauhan, K.C. Anderson, PR-924, a selective inhibitor of the immunoproteasome subunit LMP-7, blocks multiple myeloma cell growth both in vitro and in vivo, Br. J. Haematol. 152 (2011) 155–163.
[21] X.W. Dong, J.K. Zhang, L. Xu, J.X. Che, G. Cheng, X.B. Hu, L. Sheng, A.H. Gao, J. Li, T. Liu, Y.Z. Hu, Y.B. Zhou, Covalent docking modelling-based discovery of tripeptidyl epoxyketone proteasome inhibitors composed of aliphatic-heterocycles, Eur. J. Med. Chem. 164 (2019) 602–614.
[22] G. de Bruin, E.M. Huber, B.T. Xin, E.J. van Rooden, K. Al-Ayed, K.B. Kim, A.F. Kisselev, C. Driessen, M. van der Stelt, G.A. van der Marel, M. Groll, H.S. Overkleeft, Structure-based design of β1i or β5i specific inhibitors of human immunoproteasomes, J. Med. Chem. 57 (2014) 6197–6209.
[23] J. Zhang, J. Cao, L. Xu, Y. Zhou, T. Liu, J. Li, Y. Hu, Design, synthesis and biological evaluation of novel tripeptidyl epoxyketone derivatives constructed from β-amino acid as proteasome inhibitors, Bioorg. Med. Chem. 22 (2014) 2955–2965.
[24] E.M. Huber, W. Heinemeyer, G. de Bruin, H.S. Overkleeft, M. Groll, A humanized yeast proteasome identifies unique binding modes of inhibitors for the immunosubunit β5i, EMBO J. 35 (2016) 2602–2613.
[25] H.W.B. Johnson, E. Lowe, J.L. Anderl, A. Fan, T. Muchamuel, S. Bowers, D.C. Moebius, C. Kirk, D.L. McMinn, Required immunoproteasome subunit inhibition profile for anti-inflammatory efficacy and clinical candidate KZR-616 ((2 S,3 R)- N-((S)-3-(Cyclopent-1-en-1-yl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)-3-hydroxy-3-(4-methoxyphenyl)-2-((S)-2-(2-morpholinoacetamido)propanamido)propenamide), J. Med. Chem. 61 (2018) 11127–11143.
[26] D. Niewerth, J. van Meerloo, G. Jansen, Y.G. Assaraf, T.C. Hendrickx, C.J. Kirk, J.L. Anderl, S. Zweegman, G.J. Kaspers, J. Cloos, Anti-leukemic activity and mechanisms underlying resistance to the novel immunoproteasome inhibitor PR-924, Biochem. Pharmacol. 89 (2014) 43–51.