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Synthesis and Biological Evaluation of Tetrapeptide Ketones as Reversible 20S Proteasome Inhibitors

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Proteasome, a multicatalytic protease complex, has been validated as a promising therapeutic target in oncology. Carfilzomib (Kyprolis®), a tetrapeptide epoxyketone, irreversibly inhibits the chymotrypsin-like (CT-L) activity of the proteasome and has been recently approved for multiple myeloma treatment by FDA. A chemistry effort was initiated to discover the compounds that are reversibly inhibit the proteasome by replacing the epoxyketone moiety of carfilzomib with a variety of ketones as reversible and covalent warheads at the C-terminus. The newly synthesized compounds exhibited significant inhibitory activity against CT-L activity of the human 20S proteasome. When the compounds were tested for cancer cell viability, **14-8** was found to be most potent in inhibiting Molt-4 acute lymphoblastic leukemia cell line with a GI_{50} of 4.4 μM. Cytotoxic effects of **14-8** were further evaluated by cell cycle analysis and Western blotting, demonstrating activation of apoptotic pathways.

Key Words : Proteasome, Carfilzomib, Ketones, Reversible and covalent

Introduction

The proteasome, a multicatalytic protease complex, is responsible for ubiquitin-dependent non-lysosomal proteolysis of cellular proteins. Proteins that are destined for proteolysis are first tagged with polyubiquitin chains through a cascade of enzyme-catalyzed reactions and then degraded by proteasome complex in an ATP-dependent manner.[1,2] The ubiquitin-proteasome pathway plays an important role in regulating numerous cellular processes including cancer.[3] The proteasome consists of a 20S proteolytic core and two 19S regulatory caps to form a 26S complex. The 20S proteolytic core has three distinct peptidase activities; chymotrypsin-like (CT-L), trypsin-like, and caspase-like activities.[4] Of these, chymotrypsin-like activity has gained much attention since it is thought to be the rate-limiting step of proteolysis *in vitro* and *in vivo*.[5,6]

High proteasome expression has been implicated in many diseases. Blocking the proteasome activity is considered to be a promising therapeutic target in oncology since bortezomib (**1**), a dipeptide boronic acid, is approved for the treatment of patients with multiple myeloma (Figure 1).[7] In addition, carfilzomib (**2**), a structural analogue of the microbial natural product epoxomicin, has been developed and exhibited more selective inhibition of chymotrypsin-like activity of the 20S proteasome than bortezomib.[8] Carfilzomib (**2**) has been evaluated successfully in clinical trials for multiple myeloma patients and got recently approved by U.S. Food and Drug Administration (FDA).[9] Currently, its combination therapy with lenalidomide and dexamethasone is ongoing in phase III clinical trials for patients with relapsed multiple myeloma [ASPIRE trial].[10]

In spite of the clinical success of carfilzomib (**2**), there are still unmet medical needs for proteasome inhibitor therapies. Due to the instability of the epoxyketone warhead for irreversible inhibition, carfilzomib (**2**) should be administered intravenously, and requires inconvenient intravenous infusion dosing and prolonged treatment for clinical responses.[8] From this aspect, we could achieve the improvement of pharmacokinetic parameters of carfilzomib derivatives if the unstable epoxyketone warhead is displaced with more stable one such as ketones.

In this study, we report the design, synthesis, and biological evaluation of a series of 20S proteasome inhibitors that bear various ketones which are able to act as reversible and covalent warheads. The synthesized compounds were found to be active in inhibiting the CT-L activity of the 20S proteasome. In addition, we found that 14-8 is the most potent and able to induce apoptosis resulting in cancer cell death.

Results and Discussion

The starting point of our chemistry effort was carfilzomib (**2**), a tetrapeptide peptidomimetic compound with epoxyketone warhead for irreversible inhibition. We planned to synthesize the compounds having the ketones at the C-terminus for reversible and covalent inhibition instead of the original epoxyketone warheads for irreversible inhibition. A similar warhead for reversible and covalent inhibition has also been utilized in the development of Telaprevir, Hepatitis C Virus NS3·4A serine protease inhibitor.[11,12] Telaprevir contains α–ketoamide which is an electrophile as for so-called 'serine trap' warhead and found to be effective in

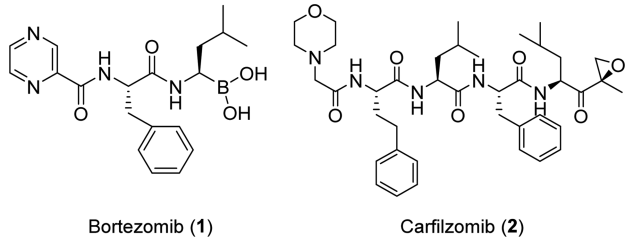


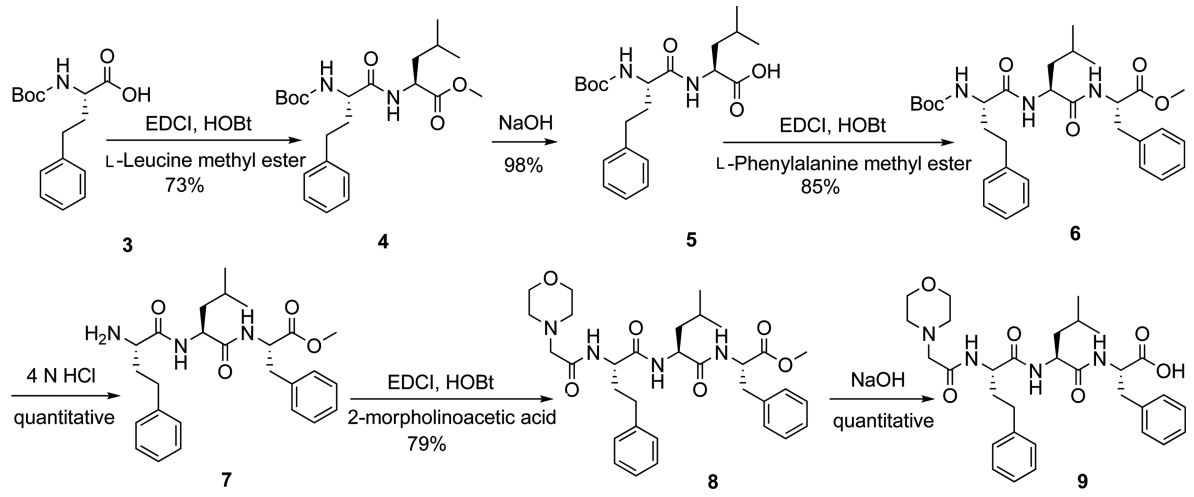
Figure 1. Structure of bortezomib (**1**) and carfilzomib (**2**).

inhibiting the viral protease.[12]

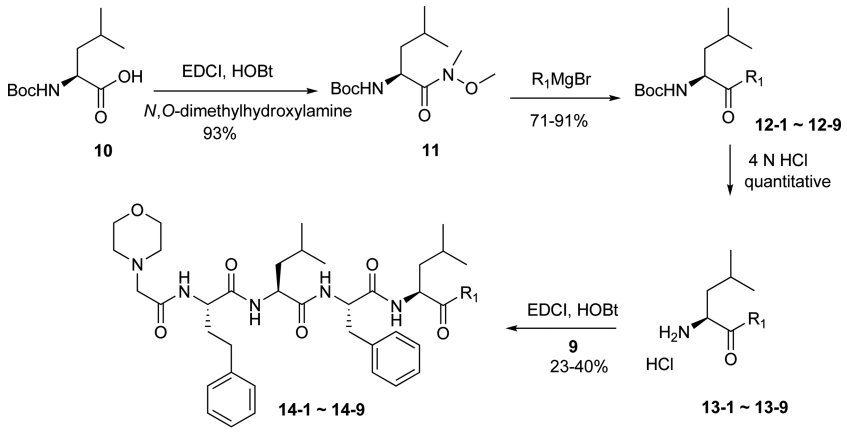
Scheme 1 and 2 summarize the procedures for synthesizing the carfilzomib derivatives having a variety of ketones for reversible inhibition. (*S*)-2-((*tert*-butoxycarbonyl)amino)-4-phenylbutanoic acid **3** was coupled with methyl L-leucine hydrochloride using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBt) in dichloromethane to provide *N*-Boc protected intermediate **4** as shown in Scheme 1. Subsequent saponification with sodium hydroxide in aqueous tetrahydrofuran afforded dipeptide **5**, followed by EDCI/HOBt mediated coupling with methyl L-phenylalanine hydrochloride to give *N*-Boc protected intermediate **6**. Removal of the *N*-Boc group with 4 N HCl gave **7** hydrochloride salt, followed by coupling with 2-morpholinoacetic acid using EDCI/HOBt to give tripeptide **8** bearing morpholine motif. Finally, further hydrolysis furnished desired acid **9** as left handed key fragment.

Synthesis of the right-hand fragment was initiated with formation of the Weinreb amide **11** from (*tert*-butoxycarbonyl)-L-leucine **10** using EDCI/HOBt in dichloromethane (Scheme 2). The Weinreb amide **11** was reacted with various Grignard reagents in THF at −78 °C to make ketone **12**, which was then treated with 4 N HCl to provide the desired aminoketone hydrochloride salt **13** ($\mathbf{R_1}$ is summarized in Table 1).[13] Finally **9** underwent EDCI/HOBt mediated amide formation with various aminoketones **13** gave the desired products of **14-1**~**14-9**. The final products were purified using silica gel column chromatography and prep-TLC if necessary.

The ability of compounds (**14-1**~**14-9**) to inhibit the CT-L activity of the human 20S proteasome was determined by using fluorogenic peptides as substrate. **14-1** and **14-2** having small aliphatic $\mathbf{R_1}$ group did not show any significant

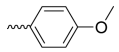
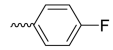
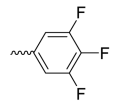
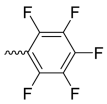
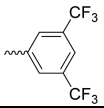


Scheme 1. Synthesis of the left hand fragments.



Scheme 2. Final coupling for the ketone inhibitors.

Table 1. Biological activities of ketone derivatives[a]

Compound	R_1	IC_{50} (μM)	GI_{50} (μM)
14-1	CH_3	> 30	> 100
14-2	C_2H_5	> 30	30.6 8.3
14-3		2.7 ± 1.1	22.3 ± 8.9
14-4		1.8 ± 0.45	15.6 ± 4.0
14-5		3.1 ± 1.1	14.7 ± 2.3
14-6		1.3 ± 0.99	24.6 ± 4.9
14-7		2.9 ± 2.1	19.8 ± 7.6
14-8		4.4 ± 3.2	4.4 ± 1.0
14-9		7.0 ± 5.4	52.7 ± 8.7

[a]IC_{50} and GI_{50} represent half maximal inhibitory concentration for proteasome activity and cell viability, respectively.

inhibitory activity for the proteasome CT-L activity. In contrast, the compounds (**14-3~14-9**) with aromatic side chain as **R_1** group were found to be effective in inhibiting CT-L activity of the proteasome whose IC_{50} values ranging from 1.3 μM to 7.0 μM (Table 1). These results strongly

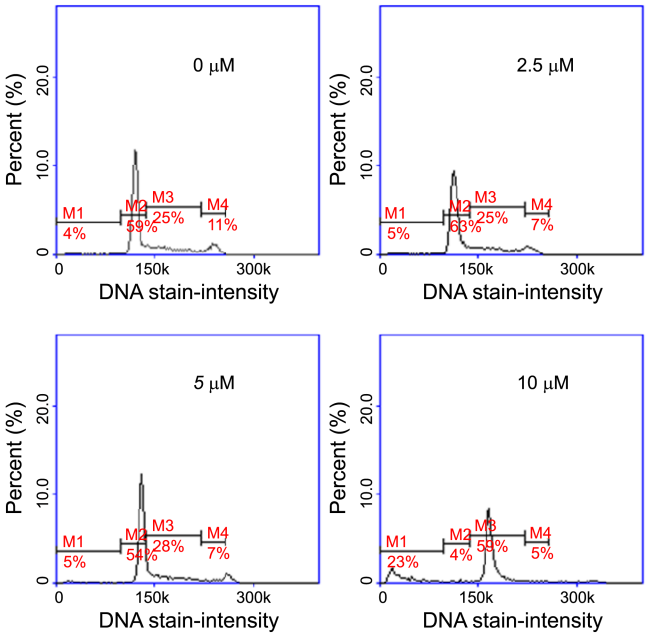


Figure 2. Apoptotic effects of compound **14-8** on Molt-4 cells. DNA content histograms of the cells treated with various concentrations of **14-8** for 48 h. The sub- G_1 population (M1) was found to be increased upon treatment of the compound from 4% (0 μM) to 24% (10 μM). M1: sub-G_1 phase, M2: G_1 phase, M3: S phase, M4: G_2/M phase.

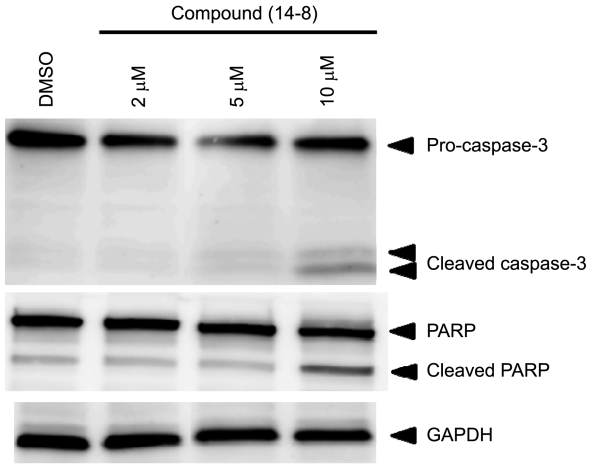


Figure 3. Western blot of the lysates from Molt-4 cells treated with compound **14-8** for 48 h. Cleaved forms of caspase-3 and PARP were significantly increased by the compound (10 μM) treatment.

suggest that bulky aromatic side chain in the ketone warhead is necessary for the inhibitory activity.

When **14-1~14-9** were treated to Molt-4 acute lymphoblastic leukemia cell line, **14-8** was found to be most effective with GI_{50} of 4.4 μM (Table 1). Cytotoxic effects of the compound were further investigated by cell cycle assays and immunoblotting. The eukaryotic cell cycle is traditionally divided into four phases, G_1, S, G_2 and M. Chromosome duplication occurs during S phase (S for DNA synthesis), and chromosome segregation and cell division occur during M phase (M for mitosis). G_1 represents the gap between M phase and S phase, while G_2 is the gap between S phase and M phase. When **14-8** is treated to the cells and causes cell-cycle arrest, cell population in G_1 or G_2/M phase would be increased, while the cell populations of sub-G_1 phase increase if the compound induces cell death.

As shown in Figure 2, the DNA content histograms clearly show that cell population in sub-G_1 phase exposed to **14-8** (23% for 10 μM) was significantly higher than that of the cells treated with DMSO only (4%), strongly indicating that the cells are undergoing apoptosis or necrosis upon compound treatment. In addition, **14-8** increased protein levels of the important apoptosis related protein markers, cleaved caspase-3 and poly ADP-ribose polymerase (PARP) in the cells, consistent with the expectation that the compound inhibits proteasome activity and induce apoptosis in the cells (Figure 3).

Conclusions

Nine carfilzomib analogues were synthesized by replacing its reactive epoxyketone with more stable ketone groups. The compounds with reversible and covalent warheads at the C-terminus were found to be effective in inhibiting the CT-L activity of the human proteasome. The compound was also effective in inhibiting cancer cell growth through apoptotic cell death. These results should provide a promising starting point for proteasome inhibitors overcoming

the disadvantages of irreversible proteasome inhibitor carfilzomib.

Experimental

Synthesis in General. ^{1}H NMR spectra were recorded on a Varian Gemini 300 & 500 spectrometer using $CDCl_3$, CD_3OD, DMSO-d_6, and acetone-d_6 as a solvent, and chemical shifts are reported in ppm downfield from that of the TMS internal standard. LCMS analysis was performed on a Waters Acquity Ultra Performance LC with waters Acquity UPLC BEH C18 1.7 micrometer 2.1 × 50 mm column and a solvent system of 10-100% acetonitrile in water with 0.1% formic acid. The mass spectrometry data were acquired on a Micromass Quattro micro mass spectrometer. All the final products display very broad ^{1}H-NMR spectra due to peptidic conformational isomers. Compound **14-8** preparation was selected to report for representative example.

***tert*-Butyl (*S*)-(1-(Methoxy(methyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (11).** To a solution of (*tert*-butoxycarbonyl)-L-leucine **10** (10 g, 43.2 mmol) in CH_2Cl_2 (150 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (9.11 g, 47.6 mmol), 1-hydroxybenzotriazole (6.42 g, 47.6 mmol) and stirred for 20 min. The reaction mixture was added with *N,O*-dimethylhydroxylamine (2.9 g, 47.6 mmol), followed by addition of triethylamine (18 mL, 130 mmol). The reaction mixture was allowed to stir overnight at room temperature. After reaction completion, solvent was removed *in vacuo* and partitioned between EtOAc and saturated $NaHCO_3$ solution. The aqueous phase was re-extracted with EtOAc. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$ and concentrated. The crude product was purified by silica gel column chromatography using hexanes/ethyl acetate (10:1) to give the desired product **11** (11.0 g, 93%) as a colorless solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.07-5.03 (m, 1 H), 4.75-4.71 (br, 1 H), 3.07 (s, 3 H), 2.95 (s, 3 H), 1.97 (s, 1 H), 1.76-1.72 (m, 2 H), 1.49 (s, 9 H), 0.96 (d, J = 6.3 Hz, 3 H), 0.93 (d, J = 6.3 Hz, 3 H); LCMS for $C_{13}H_{26}N_2O_4$ $(M + H)^+$: m/z = 275.15, Exact Mass: 274.1893.

(*S*)-*tert*-Butyl (4-Methyl-1-oxo-1-(perfluorophenyl)pentan-2-yl)carbamate (12-8). To a well stirred solution of **11** (500 mg, 1.82 mmol) in anhydrous THF at −78 °C was added dropwise pentafluorophenylmagnesium bromide solution (7.28 mL, 7.28 mmol, 1.0 M in THF). The reaction mixture was allowed to stir for 48 h at 0 °C and the reaction progress was monitored by LC-MS. After completion, the reaction mixture was quenched with 1 N HCl solution at 0 °C. The solvent was removed *in vacuo* and partitioned between EtOAc and saturated $NaHCO_3$ solution. The aqueous phase was re-extracted with EtOAc. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$ and concentrated. The crude product was purified by MPLC on silica gel column chromatography using hexanes/ethyl acetate (20:1) to give the desired product **12-8** as a colorless solid in 73% yield: $[\alpha]_D^{20}$ = +20.8 (c 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.97-4.95 (br, 1 H), 4.73 (br, 1 H), 1.79-1.75 (m, 1 H), 1.63-1.45 (m, 2 H), 1.49 (s, 9 H), 0.93 (d, J = 6.5 Hz, 6 H); LCMS for $C_{17}H_{20}F_5NO_3$ $(M + H)^+$: m/z = 382.14, Exact Mass: 381.1363. For **12-1** LCMS for $C_{12}H_{23}NO_3$ $(M + H)^+$: m/z = 230.22, Exact Mass: 229.16779; **12-2** LCMS for $C_{13}H_{25}NO_3$ $(M + H)^+$: m/z = 244.20, Exact Mass: 243.18344; **12-3** LCMS for $C_{17}H_{25}NO_3$ $(M + H)^+$: m/z = 292.19, Exact Mass: 291.1834; **12-4** LCMS for $C_{18}H_{27}NO_4$ $(M + H)^+$: m/z = 322.11, Exact Mass: 321.19401; **12-5** LCMS for $C_{17}H_{24}NFO_3$ $(M + H)^+$: m/z = 310.15, Exact Mass: 309.1740; **12-6** LCMS for $C_{17}H_{22}NF_3O_3$ $(M + H)^+$: m/z = 346.13, Exact Mass: 345.15518; **12-7** LCMS for $C_{15}H_{23}NO_3S$ $(M + H)^+$: m/z = 298.12, Exact Mass: 297.13986; **12-9** LCMS for $C_{19}H_{23}F_6NO_3$ $(M + H)^+$: m/z = 428.13, Exact Mass: 427.15821.

(*S*)-2-Amino-4-methyl-1-(perfluorophenyl)pentan-1-one hydrochloride (13-8). To a flask containing **12-8** (300 mg, 0.78 mmol) in ethyl acetate (2 mL) was added 4 N HCl (4 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 hr, concentrated *in vacuo* and used directly without purification as solid (quantitative): $[\alpha]_D^{20}$ = +12.0 (c 1.0, $CHCl_3$); LCMS for $C_{12}H_{12}F_5NO$ $(M + H)^+$: m/z = 282.10, Exact Mass: 281.0839.

(*S*)-4-Methyl-*N*-((*R*)-1-(((*R*)-4-methyl-1-oxo-1-(perfluorophenyl)pentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)-2-((*S*)-4-phenyl-2-(2-(tetrahydro-2*H*-pyran-4-yl)acetamido)-butanamido)pentanamide (14-8). To a solution of **9** (250 mg, 0.44 mmol) in CH_2Cl_2 (5 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (169 mg, 0.88 mmol), 1-hydroxybenzotriazole (119 mg, 0.88 mmol) and stirred for 20 min. The reaction mixture was added with **13-8** (149 mg, 0.53 mmol), followed by addition of triethylamine (0.20 mL, 1.32 mmol). The reaction mixture was allowed to stir overnight at room temperature. The reaction mixture was concentrated and partitioned with EtOAc and saturated $NaHCO_3$ solution. The aqueous phase was re-extracted with EtOAc. The combined organic phases were washed with brine, dried over anhydrous $MgSO_4$ and concentrated. The crude product was purified by silica gel column chromatography using hexanes/ethyl acetate/methanol (1:3:0.5) to give the desired product **14-8** as a colorless solid in 35% yield: $[\alpha]_D^{20}$ = +98.5 (c 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21-7.11 (m, 10 H), 3.75 (br, 1 H), 3.64-3.59 (m, 3 H), 2.63-2.58 (br, 4 H), 2.02-1.93 (br, 4 H), 1.85-1.74 (br, 4 H), 1.64-1.41 (br, 10 H), 1.25 (br, 6 H), 0.96-0.87 (m, 11 H); LCMS for $C_{44}H_{53}F_5N_4O_6$ $(M + H)^+$: m/z = 829.28, Exact Mass: 828.3885. For **14-1** LCMS for $C_{38}H_{55}N_5O_6$ $(M + H)^+$: m/z = 678.50, Exact Mass: 677.41523; **14-2** LCMS for $C_{40}H_{58}N_4O_6$ $(M + H)^+$: m/z = 692.50, Exact Mass: 690.43564; **14-3** LCMS for $C_{43}H_{55}N_5O_6$ $(M + H)^+$: m/z = 740.50, Exact Mass: 739.43088; **14-4** LCMS for $C_{45}H_{60}N_4O_7$ $(M + H)^+$: m/z = 769.90, Exact Mass: 768.44620; **14-5** LCMS for $C_{44}H_{57}FN_4O_6$ $(M + H)^+$: m/z = 794.50, Exact Mass: 758.50; **14-6** LCMS for $C_{44}H_{55}F_3N_4O_6$ $(M + H)^+$: m/z = 794.50, Exact Mass: 792.40737; **14-7** LCMS for $C_{42}H_{56}N_4O_6S$ $(M + H)^+$: m/z = 746.80, Exact Mass: 744.39206; **14-9** LCMS for $C_{45}H_{55}F_6N_5O_6$ $(M + H)^+$: m/z = 876.30, Exact Mass: 875.40565.

Proteasome Assay. The proteasome activity was deter-

mined at 25 °C in a buffer solution containing 20 mM Tris-Cl, pH 7.0, 0.5 mM EDTA, 0.03% SDS and synthetic fluorogenic peptide substrate, succinyl-Leu-Leu-Val-Tyr-AMC, by measuring the AMC product formation according to the procedures as described previously with a slight modification.[6] The reaction was initiated by the addition of purified 20S proteasomes. Reaction rate was determined based on the reaction velocity measured between 30 and 40 min.

WST-1 Cytotoxicity Assay. Cytotoxic effects were evaluated with the reagent WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium]. WST-1 produces a highly water soluble formazan upon metabolically active cells, allowing a direct colorimetric measurement of cell viability. Cells were seeded on 96-well plates at a density of 1×10^4 cells per well and treated with the test compounds at various concentrations. After 48 h, cells were incubated with WST-1 for 1 h and the resulting absorbance was measured at 450 nm.

Cell Cycle Analysis. The cell cycles of Molt-4 cells treated with compounds were analyzed by using NucleoCounter NC-250 system (Chemometec, Denmark). First, the cells were resuspended in lysis buffer (solution 10) containing nuclear stain dye DAPI. Subsequently, 2×10^6 cells were harvested by centrifugation and further treated with stabilization buffer (solution 11). Cellular fluorescence is quantified and DNA content histogram was obtained by a two-step cell cycle analysis protocol provided by the company.

Immunoblotting. Cells were seeded at 70% confluence in 6-well plates, treated with the compounds next day and incubated for 48 h. To prepare the whole cell lysates, cells were washed twice with PBS and lysed with RIPA buffer containing protease inhibitors. Subsequently, cell lysates were electrophoretically separated on a 10% SDS-polyacrylamide gel. Proteins were transferred onto PVDF membranes (polyvinyidenfluoride, Millipore, MA, USA) overnight at 4 ºC. Membranes were blocked with PBS containing 5% skim milk and 0.1% Tween 20, pH 7.4 for 1 h and then incubated sequentially with primary and secondary antibodies diluted in blocking buffer. Protein bands were detected with chemiluminescence detection system (Cell Signaling, MA, USA) on a LAS-3000 (Fuji, Japan).

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