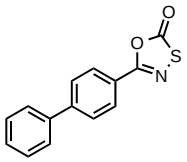
Supporting information

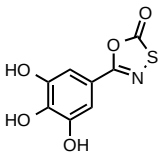
Materials and Methods

Materials: All chemicals for synthesis were purchased from Bachem, Sigma, or AnaSpec and used as received. Suc-LLVY-AMC, Z-LLE-AMC and Z-LRR-AMC were from Boston Biochem (Cambridge, MA). Ac-ANW-AMC was a kind gift from Dr. Christopher Tsu at Millennium Pharma Inc. Human immunoproteasome (PBMCs) and human constitutive proteasome (RBCs) were from Boston Biochem Inc. Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay kit and CellTiter-Glo® Luminescent Cell Viability Assay were from Promega (Madison, WI). Karpas-1106P cells were from Sigma (catalogue no. 06072607), and the cells were cultured in RPMI, 2mM glutamine, 20% fetal bovine serum, and 100 units/ml penicillin & 100 μg/ml streptomycin (all from Invitrogen). Monoclonal antibody against ubiquitin was from Enzo Life Sciences (catalogue no. BML-PW8810-0100). IRDye 800CW goat anti-mouse IgG1 Specific was from Li-Cor (Lincoln, NE).

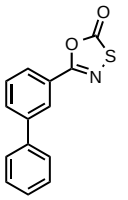
Chemicals and Spectroscopy. Unless otherwise stated, all commercially available materials were purchased from Aldrich, TCI, or other vendors and were used as received. All non-aqueous reactions were performed under argon. Microwave-based reactions were performed on a CEM Discover Microwave irradiator, using the dynamic heating mode with a power limit of 250 W and pressure limit of 17 psi. ^{1}H- and ^{13}C- NMR spectra were acquired on a Bruker DRX-500 spectrometer. Chemical shifts δ are expressed in parts per million, with either tetramethylsilane (TMS) or the solvent resonance as an internal standard (TMS, ^{1}H: 0 ppm; chloroform, 13C: 77.0 ppm; DMSO-d_6, 1H: 2.5 ppm; ^{13}C: 39.5 ppm). NMR data are reported as following: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), integration, and coupling constant. Low Resolution Mass spectra (LRMS) were obtained on a Perkin Elmer SCIEX API 100 mass spectrometer and High Resolution Mass Spectra (HRMS) were obtained on Waters LCT Premier/XE. Not all 1,3,4-oxathiazol-2-ones gave HRMS data. 1,3,4-oxathiazol-2-ones compounds were synthesized as previously reported (Lin, G. et al. Nature, 2009, 8357). Spectroscopic data of new compounds are reported below.



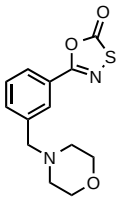
HT2004: 97% yield; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.03 (d, *J* = 8.1 Hz, 2H), 7.71 (d, *J* = 8.1 Hz, 2H), 7.67-7.60 (m, 2H), 7.52-7.45 (m, 2H), 7.45-7.39 (m, 1H); ^{13}C NMR ($CDCl_3$) δ174.0, 157.4, 145.5, 139.6, 129.2, 128.5, 128.0, 127.7, 127.3, 124.6. LRMS (M + Na^+) m/z: calcd 278.0; found 278.0.



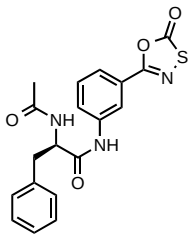
HT2050: 97% yield; ^{1}H NMR (500 MHz, MeOD) δ 6.99 (s, 2H); ^{13}C NMR (MeOD) δ175.6, 159.3, 147.2, 122.4, 114.1, 107.7. HRMS (M – H^+) m/z: calcd 225.9810; found 225.9801.



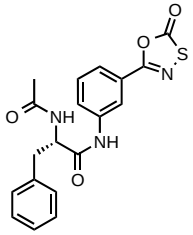
HT2068: 76% yield; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.22-8.19 (m, 1H), 7.95 (ddd, *J* = 1.1, 1.7, 7.8 Hz, 1H), 7.79 (ddd, *J* = 1.2, 1.7, 7.8 Hz, 1H), 7.65-7.60 (m, 2H), 7.59-7.54 (m, 1H), 7.51-7.46 (m, 2H), 7.44-7.39 (m, 1H); ^{13}C NMR ($CDCl_3$) δ174.0, 157.5, 142.4, 139.7, 131.4, 129.7, 129.2, 128.2, 127.3, 126.4, 126.3, 126.2. LRMS (M + Na^+) m/z: calcd 278.0; found 278.0.



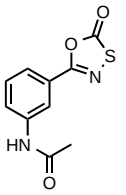
HT2090: 7% yield; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.99-7.93 (m, 1H), 7.89-7.84 (m, 1H), 7.58-7.50 (m, 1H), 7.48-7.40 (m, 1H), 3.74-3.70 (m, 4H), 3.55 (s, 2H), 2.52-2.40 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 173.9, 157.7, 139.6, 133.5, 129.2, 128.1, 126.5, 126.1, 67.1, 63.0, 53.8. HRMS (M + H^+) m/z: calcd 279.0803; found 279.0799.



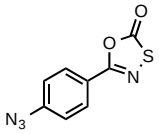
HT2106: 67 % yield; ^{1}H NMR (500 MHz, DMSO) δ 10.49 (s, 1H), 8.35 (d, *J* = 7.9 Hz, 1H), 7.92-7.86 (m, 2H), 7.78 (d, *J* = 8.6 Hz, 2H), 7.32-7.24 (m, 4H), 7.22-7.16 (m, 1H), 4.66 (ddd, *J* = 5.3, 7.9, 9.5 Hz, 1H), 3.03 (dd, *J* = 5.3, 13.8 Hz, 1H), 2.86 (dd, *J* = 9.5, 13.8 Hz, 1H), 1.80 (s, 3H); ^{13}C NMR (DMSO) δ 173.9, 171.1, 169.4, 156.5, 142.7, 137.5, 129.1, 128.2, 128.1, 126.4, 124.4, 120.1, 119.2, 55.1, 37.5, 22.3. HRMS (M - H^+) m/z: calcd 382.0862; found 382.0866.



HT2111: 13% yield; ^{1}H NMR (500 MHz, DMSO) δ 10.54 (s, 1H), 8.36 (d, *J* = 7.9 Hz, 1H), 7.84-7.72 (m, 4H), 7.30-7.24 (m, 4H), 7.22-7.16 (m, 1H), 4.65 (ddd, *J* = 5.4, 8.8, 9.4 Hz, 1H), 3.02 (dd, *J* = 5.4, 13.7 Hz, 1H), 2.85 (dd, *J* = 9.4, 13.7 Hz, 1H), 1.80 (s, 3H); ^{13}C NMR (DMSO) δ 171.3, 169.4, 143.0, 137.4, 133.3, 129.1, 128.1, 126.4, 119.3, 119.0, 105.1, 55.1, 37.5, 22.3. LRMS (M + Na^{+}) m/z: calcd 406.1; found 406.1.



HT2210: 69% yield; ^{1}H NMR (500 MHz, DMSO) δ 10.22 (s, 1H), 8.32-8.27 (m, 1H), 7.79-7.73 (m, 1H), 7.62-7.55 (m, 1H), 7.52-7.47 (m, 1H), 2.07 (s, 3H); ^{13}C NMR (DMSO) δ 173.8, 168.7, 156.6, 140.0, 129.8, 126.0, 122.7, 121.5, 117.0, 24.0. HRMS (M + Cl^{-}) m/z: calcd 270.9944; found 270.9942.



NA1129: 92% yield; ^{1}H NMR (500 MHz, DMSO) δ 7.98-7.91 (m, 2H), 7.34-7.27 (m, 2H); ^{13}C NMR (DMSO) δ 206.4, 173.8, 156.1, 143.8, 129.0, 122.1, 119.9. LRMS (M + Na^{+}) m/z: calcd 243.0; found 243.2.

Kinetics

Assays were conducted on a SpectraMax Gemini plate-reader from Molecular Devices (Sunnyvale, CA). In a black 96-well plate, 100 µL of pre-warmed assay mixture containing 0.4 nM Hu i-20S or 0.2 nM Hu c-20S, 25 µM suc-LLVY-AMC, 0.02% SDS in buffer (50 mM HEPES, 0.5 mM EDTA, pH7.4) was added to the wells that contained 1 µL inhibitor at 100x indicated concentrations in DMSO. The reaction progress of each well was recorded by monitoring fluorescence at 460 nm (λ_{ex} = 360 nm) for 120 minutes at 37˚C. Values of k_{obs} were derived from the fit of data to equation (I) in Prism (GraphPad Software, Inc. La Jolla, CA), and then were plotted against inhibitor concentrations to obtain k_{inact} and K_I for each inhibitor.

Measurement of IC50s.

0.5 μL of oxathiazolones of series dilution from 20 mM in DMSO were spotted at the bottom of the wells of a black 96-well plate. 50 μL of 2x enzyme solution (0.8 nM Hu i-20S) in the above described HEPES buffer were dispensed into each well and incubated at room temperature for 30 minutes. 50 μL of 2x substrate solution (50 μM Suc-LLVY-AMC for β5i and β5c, 50 μM Z-VLR-AMC for β2i, and 50 μM Ac-PAL-AMC [gift from Dr. Christopher Tsu at Millennium Pharma Inc.] for β1i) were added and spin at 1000 rpm for 1 minute. In the case of β2i activity, PA28 (final conc. 2 nM) was used as activator to replace SDS. The reaction progress of each well was recorded by monitoring fluorescence at 460 nm (λ_{ex} = 360 nm) for 120 minutes at 37˚C in the plate reader for 2 hours. The progresses of the reaction in each well were recorded and the initial linear ranges were used to calculate the velocity. IC50s were calculated with Prism GraphPad.

Inhibition of β2i and β1i activity of the human i-20S.

0.5 μL of oxathiazolones at 20 mM in DMSO were spotted at the bottom of the wells of a black 96-well plate. 50 μL of 2x enzyme solution (0.8 nM Hu i-20S) in HEPES buffer were dispensed into each well and incubated at room temperature for 30 minutes. 50 μL of 2x substrate solution (50 μM Z-VLR-AMC for β2i, and 50 μM Ac-PAL-AMC for β1i). The progresses of the reaction in each well were recorded and the initial linear ranges were used to calculate the velocity.

Determination of T1/2 of HT2210 and HT2106 in RPMI

Solutions of HT2210 or HT2106 in RPMI were prepared at 75 μM, respectively, with pyrazinamide (100 μM) as an internal control, and incubated at room temperature. Aliquots were taken at 0, 10, 30, 60, 120, 180, and 240 minutes for UPLC/MS analysis. The remaining amount of compounds calculated from peak areas of HT2210 or HT2106 were normalized to that of the internal control. The fit of the % remaining versus time yielded exponential decay. The rates (k) of the decay were calculated with Prism and the half-lives by equation t1/2 = 0.693 / k.

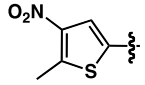
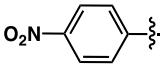
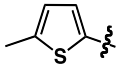
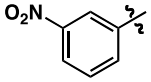
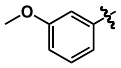
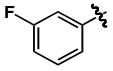
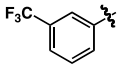
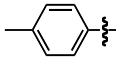
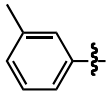
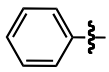
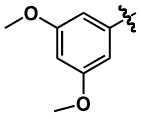
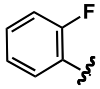
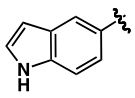
Inhibition of 26S chymotryptic-like activity in intact cells

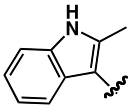
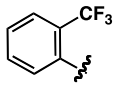
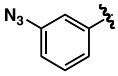
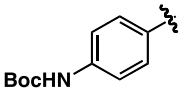
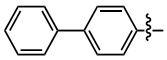
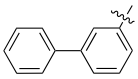
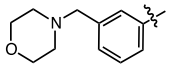
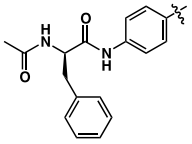
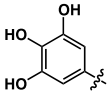
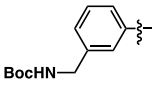
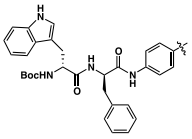
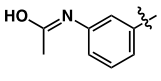
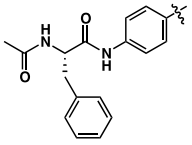
Karpas 1106p cells at 80,000 cells per well were plated in 96-well plates and incubated with compound at indicated concentrations for 3 h at 37℃. After spinning at 1200 rpm for 5 minutes, supernatant was carefully removed without disturbing the cell pellet. Cells were resuspended in media, and proteasome activities in each well were measured *in situ* by monitoring hydrolysis of the β5 (chymotrypsin-like) substrate Suc-LLVY-(D)-aminoluciferin in the presence of luciferase using the Proteasome-Glo™ assay reagents according to the manufacturer's instructions (Promega). Luminescence was measured using a Molecular Devices SpectraMax M5 plate reader. IC_{50}s were calculated by fitting the data to equation of sigmoidal dose-response with variable slope using PRISM.

Accumulation of polyubiquitinated proteins

Two million Karpas cells in 2 mL were treated with HT2210 and HT2106 at 2.5 µM or 25 µM, respectively for 4 hours at 37˚C, 5% CO_2. DMSO was used as vehicle control, and Bortezomib as positive control at 50 nM and 500 nM. DMSO concentration was maintained at 0.5%. Harvested cells were washed with PBS twice, and lysed in 50 mM Tris, pH7.5 on ice for 30 minutes. 30 µg of lysate proteins were used for SDS page (4-20%) and analyzed by western-blot with antibody against ubiquitin.

Table S1. IC50s of oxathiazolones against human c-20S β5c and i-20S β5i and percentage of inhibition of the hu i-20S β1iand β2i.

ID	R	IC50 (μM)		% Inhibition @ 100 μM	
		Hu20S β5i	Hu20S β5	β1i	β2i
HT1171		0.22	7.9	89 (2.9 μM)[a]	100 (3.1 μM)[a]
HT1041		0.78	32.6	58	56
HT1044		0.093	5.4	90 (4.6 μM)[a]	71
HT1042		0.49	39.3	76	52
HT1043		3.47	>100	49	43
HT1054		3.18	>100	21	15
HT1071		3.03	68.7	74	55
HT1086		0.25	37.5	58	43
HT1117		0.50	6.2	64	44
HT1118		0.45	10.9	53	59
HT1146		0.15	10.6	71	67
HT1147		2.32	>100	15	27
HT1197		0.16	10.6	64	56
HT1213		1.32	60	42	33.5

HT1214		0.86	>100	25	30.4
HT1223		0.15	11.6	41	52
HT1228		0.49	26.3	50	39
HT1278		4.28	70.0	24	29
HT2004		2.68	>100	12	1.2
HT2068		0.15	87.1	37	52
HT2090		0.36	60.2	21	50
HT2106		0.24	>100	24	52
HT2050		0.13	33.1	42	89 (9.5 μM)[a]
HT2170		0.64	66.9	18	35
HT2197		1.75	>100	40	23
HT2210		0.97	25.3	30	47
HT2111		9.97	>100	16	22

[a] IC50s against hu i-20S β1i and β2i.

Figure S1. Comparison of amino acid residues that are involved in the conformational changes in the Mtb20S upon inhibition by oxathiazolones in Mtb20S, hu c-20S β1, β2, β5, and hu i-20S β1i, β2i, β5i. Amino acid residues whose hydrogen bonds are broken (A) and formed (B) following the inactivation of the Mtb20S by oxathiazolones and corresponding amino acids residues in three active sites of the hu i-20S and hu c-20S.