Supporting Information:

Design and Synthesis of an Orally Bioavailable and Selective Peptide Epoxyketone Proteasome Inhibitor (PR-047)

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Contents:

Biological and analytic assays:

20S proteasome and cellular proteasome activity assays; MES cell viability assays: Assays were preformed as described by Demo et al. (reference 22).

Assays to evaluate stability in simulated intestinal fluid (SIF) and simulated gastric fluid (SGF): SIF was prepared by dissolving 0.68 grams of pancreatin (Mallinckrodt Baker, Inc) in 100 mL of 50 mM potassium phosphate buffer (pH 7.4). SGF was prepared by dissolving 3.2 g of pepsin (355U/mg, Sigma) in 1000 mL water containing 2.0 g of sodium chloride (EMD Chemicals Inc.,) and 7.0 mL of concentrate hydrochloric acid (Sigma) (pH about 1.2). A 480-µL aliquot of the SIF buffer or SGF solution was loaded into a well of a Costar 2-mL 96-well plate (Corning Inc., Corning, NY). 20 µL of a test article solution at 100 µM was added to give a final concentration of the test article at 4 µM. The mixture was incubated at 37 °C with gentle agitation. An aliquot of 100-µL of the reaction mixture was taken at 0, 15, and 45 minutes and quenched using 200 µL acetontrile containing 1 µM compound **31** as the internal standard. After quenching, the mixture was vortexed, centrifuged and filtered through a membrane (Pall Corporation AcroPrep p6 Filter Plate, 0.45 µm). 10-µL of the resulting solution was injected into a SCIEX API3000 or API3200 LC/MS/MS equipped with an electrospray ionization source for determination of the test article. Percentage remaining of the test article at the incubation times of 15 and 45 minutes relative to 0 minute was calculated using peak area ratio of the test article versus the internal standard.

Liver microsomal assays: 10 mL of a microsomal solution was mixed with 200 µL of 50 mM NADPH solution to prepare a microsomes-NADPH solution. 500 µL of the microsomes-NADPH solution was pre-warmed at 37 °C for 5 minutes in Costor 2-mL

96-well plate (Corrning Inc., Corning, NY). 5 µL of a test article solution at 100 µM was then added to initiate the reaction. The final incubation mixture contained 1 mg/mL liver microsomes, 1 µM test article, 1 mM NADPH, 0.1% DMSO and 1% acetonitrile in 100 mM phosphate buffer at pH=7.4. The incubation mixture was kept at 37 °C and 40-µL aliquots were taken at times of 0, 10, 20, 30, 60 and 90 minutes. In each aliquot, the reaction was quenched using 120 µL acetontrile containing 1 µM compound **31** as the internal standard. After quenching, the mixtures were vortexed and centrifuged. The supernatant was transferred and filtered through a membrane (Pall Corporation AcroPrep p6 Filter Plate, 0.45 µm). 10-µL of the resulting solution was injected into a SCIEX API3000 or API3200 LC/MS/MS equipped with an electrospray ionization source for determination of a test article at different times of incubation. The peak area ratio of a test article versus the internal standard was used in the calculation of rate of disappearance of a test article. In vitro liver extraction ratio was calculated from the rate of disappearance using the approach developed by Obach (see reference 36).

Pharmacokinetics and pharmacodynamics: For pharmacokinetic analysis, compounds were administered to mice, rats, and dogs (n = 3 – 4 per dose group) in a vehicle 10 - 20% (v/v) PS80 and 10% (v/v) EtOH in 50 mmol/L sodium citrate buffer (pH 3.5). At selected time points after administration, blood samples were collected and processed to plasma, compound levels were determined by LC/MS. For pharmacodynamic (proteasome inhibition) assays, blood and tissue samples (adrenal, brain, and liver) were collected at various times post dose, processed to cell lysates and proteasome activity was measured using LLVY-AMC as a substrate, as preciously described.[22]

Animal efficacy studies: Tumors were established by s.c. injection of RL cells (passage number <9 and viability >95% at the time of implantation) in the right flank of BNX mice (n = 8 - 10 per group). For RL studies, cell suspensions containing 1 x 10^7 cells in a volume of 0.1 mL were injected. Mice were randomized into treatment groups and dosing initiated when tumors reached ~100 mm^3 (RL). For CT-26 studies, BALB/c mice (n = 10/group) were challenged with 2 x 10^5 cells/mouse in the right flank. Dosing began on Day 3 post tumor challenge. Tumors were measured thrice weekly by recording the longest perpendicular diameters and tumor volumes were calculated using the equation V (in mm^3) = (length X $width^2$)/2.

Solubility assay: Qualitative solubility assessments were made for all compounds to determine suitability for oral dosing. Compounds were evaluated for solubility ≥1 mg/ml in oral dosing vehicle (10% EtOH, 10% PS80 in citrate buffer pH 3.5) by visual inspection. For quantitative solubility assessments, 5-15 mg solid samples were weighed and shaken with 1.00 mL of vehicle (10% EtOH, 10% PS80 in citrate buffer pH 3.5) at 25 0C for 12 hours. The resulting samples were filtered and the filtrate was diluted 4-fold with a mixture of MeOH/water (1/1 ratio). Each compound was run in duplicate. The standard samples (0.25 mg/mL solution) were prepared from 4-fold dilution of 1.00 mg/mL methanol solution with a mixture of MeOH/water (1/1 ratio). HPLC analysis was performed with filtered 0.1 M sodium perchlorate (pH=3.1) solution as Mobile Phase A and acetonitrile as Mobile Phase B using the following chromatography system: 4.6-mm x 150-mm column containing C18 packing (Waters Symmetry C18 3.5 µM column, part #: WAT200632) maintained at 30˚C with a column heater and equipped with a 214-nm detector.

Analytical LCMS method for purity assessment:

Column	Eclipse XDB-C8 150-4.6 mm		
Column temperature	50 ^{0}C		
Run time	27 min		
Flow rate	1.0 mL/min		
Mobile phase	A: water w/ 0.01% HOAc		
	B: MeCN w/ 0.01% HOAc		
Gradient	Time	% A	% B
	0.0	70	30
	20.0	30	70
	23.0	30	70
	23.1	2	98
	24.0	2	98
	24.1	70	30
	27.0	70	30

Results:

(2*S*)-4-methyl-N-[(1*S*)-1-{[(2*S*)-4-methyl-1-[(2*R*)-2-methyloxiran-2-yl]-1-oxopentan-2-yl]carbamoyl}-2-phenylethyl]-2-[2-(morpholin-4-yl)acetamido]pentanamide (5): ^{1}H-NMR (300.05 MHz, $CDCl_3$) δ: 0.88 (*m*, 12H, $2(C\underline{H}_3)_2CH$), 1.22 (*m*, 2H, $2C\underline{H}_2CH(CH_3)_2$), 1.48 (*s*, 3H, $C\underline{H}_3$-oxirane), 1.49-1.74 (*m*, 4H, $2C\underline{H}(CH_3)_2$ & $2C\underline{H}_2CH(CH_3)_2$), 2.52 (*br*, 4H, $C\underline{H}_2NC\underline{H}_2$ of morpholine), 2.87 (*d*, *J*=4.8 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.01-3.10 (*m,* 4H, $C\underline{H}_2Ph$ & $NC\underline{H}_2$), 3.24 (*d*, *J*=5.1 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.70-3.75 (*m*, 4H, $C\underline{H}_2OC\underline{H}_2$ of morpholine), 4.33 (*q*, *J*=8.7, 16.5 Hz, 1H, $C\underline{H}NH$), 4.50-4.72 (*m*, 2H, $2C\underline{H}NH$), 6.44 (*d*, *J*=8.1 Hz, 1H, $N\underline{H}$), 6.90 (*d*, *J*=7.8 Hz, 1H, $N\underline{H}$), 7.16-7.29 (*m*, 5H, $C_6\underline{H}_5$), 7.53 (*d*, *J*=7.5 Hz, 1H, $N\underline{H}$). ^{13}C-NMR (75.46 MHz, $CDCl_3$) δ: 16.91, 21.47, 22.13, 23.10, 23.56, 25.12, 25.28, 37.75, 40.20, 40.43, 50.36, 51.80, 52.57, 53.86, 54.45, 59.21, 61.42, 66.74, 127.34, 129.55, 136.69, 170.86, 170.87, 171.96, 208.30.

(2*S*)-4-methyl-2-[(5-methyl-1,2-oxazol-3-yl)formamido]-N-[(1*S*)-1-{[(2*S*)-4-methyl-1-[(2*R*)-2-methyloxiran-2-yl]-1-oxopentan-2-yl]carbamoyl}-2-phenylethyl]pentanamide (**11**): ^{1}H-NMR (300.05 MHz, $CDCl_3$) δ: 0.88 (*m*, 12H, 2($C\underline{H}_3$)$_2$CH), 1.20 (*m*, 2H, 2$C\underline{H}_2$CH(CH_3)$_2$), 1.48 (*s*, 3H, $C\underline{H}_3$-oxirane), 1.49-1.74 (*m*, 4H, 2C$\underline{H}$(CH_3)$_2$ & 2$C\underline{H}_2$CH(CH_3)$_2$), 2.50 (*s*, 3H, $C\underline{H}_3$-isoxazole), 2.88 (*d*, *J*=4.5 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.03 (*d*, *J*=7.2 Hz, 2H, $C\underline{H}_2$Ph), 3.25 (*d*, *J*=5.1 Hz, 1H, $C\underline{H}_2$ of oxirane), 4.49-4.55 (*m*, 2H, 2C$\underline{H}$NH), 4.62 (*q*, *J*=6.6, 14.7 Hz, 1H, C$\underline{H}$NH), 6.24 (*d*, *J*=8.4 Hz, 1H, N$\underline{H}$), 6.39 (*d*, *J*=0.3 Hz, 1H, $\underline{H}$-isoxazole), 6.66 (*d*, *J*=8.1 Hz, 1H, N$\underline{H}$), 7.03 (*d*, *J*=7.8 Hz, 1H, N$\underline{H}$), 7.15-7.26 (*m*, 5H, $C_6\underline{H}_5$). ^{13}C-NMR (75.46 MHz, $CDCl_3$) δ: 12.62, 16.91, 21.46, 22.05, 23.09, 23.55, 24.97, 25.22, 40.22, 40.61, 52.06, 52.57, 54.30, 59.25, 59.43, 101.65, 127.18, 128.80, 129.45, 136.42, 158.16, 159.61, 170.67, 171.23, 171.68, 208.09.

(2*S*)-4-methyl-N-[(1*S*)-1-{[(2*S*)-4-methyl-1-[(2*R*)-2-methyloxiran-2-yl]-1-oxopentan-2-yl]carbamoyl}-2-phenylethyl]-2-{[5-(morpholin-4-ylmethyl)-1,2-oxazol-3-yl]formamido}pentanamide (**28**): ^{1}H-NMR (300.05 MHz, $CDCl_3$) δ: 0.88 (*m*, 12H, 2($C\underline{H}_3$)$_2$CH), 1.18 (*m*, 1H, $C\underline{H}_2$CH(CH_3)$_2$), 1.48 (*s*, 3H, $C\underline{H}_3$-oxirane), 1.40-1.69 (*m*, 5H, 2C$\underline{H}$(CH_3)$_2$ & 3$C\underline{H}_2$CH(CH_3)$_2$), 2.52 (*t*, *J*=4.8 Hz, 4H, $C\underline{H}_2$N$C\underline{H}_2$ of morpholine), 2.87 (*d*, *J*=5.1 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.00 (*d*, *J*=7.2 Hz, 2H, $C\underline{H}_2$Ph), 3.23 (*d*, *J*=5.1 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.70-3.73 (*m*, 6H, $C\underline{H}_2$O$C\underline{H}_2$ of morpholine & N$C\underline{H}_2$), 4.48-4.61 (*m*, 2H, 2C$\underline{H}$NH), 4.65 (*q*, *J*=6.9, 14.4 Hz, 1H, C$\underline{H}$NH), 6.36 (*d*, *J*=8.1 Hz, 1H, N$\underline{H}$), 6.62 (*s*, 1H, $\underline{H}$-isoxazole), 6.80 (*d*, *J*=7.5 Hz, 1H, N$\underline{H}$), 7.10-7.26 (*m*, 6H, N$\underline{H}$ & $C_6\underline{H}_5$). ^{13}C-NMR (75.46 MHz, $CDCl_3$) δ: 16.89, 21.50, 22.077, 23.11, 23.54, 24.95, 25.25, 38.08, 40.27, 40.82, 50.27, 52.01, 52.53, 52.54, 53.44, 53.74, 54.35, 59.20, 66.97, 103.53, 127.14, 128.75, 129.48, 136.44, 158.09, 159.22, 170.69, 171.27, 171.28, 208.14.

(2*S*)-2-[(2*S*)-3-methoxy-2-[(5-methyl-1,2-oxazol-3-yl)formamido]propanamido]-N-[(2*S*)-4-methyl-1-[(2*R*)-2-methyloxiran-2-yl]-1-oxopentan-2-yl]-3-phenylpropanamide (**29**): ^{1}H-NMR (300.05 MHz, $CDCl_3$) δ: 0.88 (*dd*, *J*=6.0, 12.5 Hz, 6H, $(C\underline{H}_3)_2CH$), 1.18 (*m*, 1H, $C\underline{H}_2CH(CH_3)_2$), 1.43 (*m*, 2H, $C\underline{H}(CH_3)_2$ & $C\underline{H}_2CH(CH_3)_2$), 1.48 (*s*, 3H, $C\underline{H}_3$-oxirane), 2.48 (*d*, *J*=0.3 Hz, 3H, $C\underline{H}_3$-isoxazole), 2.88 (*d*, *J*=4.8 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.00 (*dd*, *J*=6.3, 13.8 Hz, 1H, C$\underline{H}$Ph), 3.16 (*dd*, *J*=6.3, 13.8 Hz, 1H, C$\underline{H}$Ph), 3.28 (*d*, *J*=5.1 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.33 (*s*, 3H, $OC\underline{H}_3$), 3.57 (*dd*, *J*=6.9, 9.3 Hz, 1H, $C\underline{H}_2OCH_3$), 3.84 (*dd*, *J*=4.2, 9.3 Hz, 1H, $C\underline{H}_2OCH_3$), 4.52-4.71 (*m*, 3H, 3C$\underline{H}$NH), 6.35 (*d*, *J*=0.9 Hz, 1H, $\underline{H}$-isoxazole), 6.41 (*d*, *J*=8.1 Hz, 1H, N$\underline{H}$), 6.69 (*d*, *J*=7.8 Hz, 1H, N$\underline{H}$), 7.13-7.26 (*m*, 5H, $C_6\underline{H}_5$), 7.54 (*d*, *J*=6.9 Hz, 1H, N$\underline{H}$). ^{13}C-NMR (75.46 MHz, $CDCl_3$) δ: 12.59, 16.88, 21.46, 23.55, 25.14, 25.15, 37.54, 40.22, 50.19, 52.56, 52.95, 54.26, 59.23, 59.41, 71.25, 76.85, 101.54, 127.29, 128.91, 129.54, 136.19, 136.20, 136.21, 136.21, 158.14, 159.67, 169.12, 170.54, 171.64, 208.28.

(2*S*)-3-methoxy-2-[(2*S*)-3-methoxy-2-[(5-methyl-1,2-oxazol-3-yl)formamido]propanamido]-N-[(2*S*)-4-methyl-1-[(2*R*)-2-methyloxiran-2-yl]-1-oxopentan-2-yl]propanamide (**49**): ^{1}H-NMR (300.05 MHz, $CDCl_3$) δ: 0.90 (*d*, *J*=6.3 Hz, 6H, $(C\underline{H}_3)_2CH$), 1.26 (*m*, 1H, $C\underline{H}_2CH(CH_3)_2$), 1.48 (*s*, 3H, $C\underline{H}_3$-oxirane), 1.51 (*m*, 1H, $C\underline{H}_2CH(CH_3)_2$), 1.61 (*m*, 1H, $C\underline{H}(CH_3)_2$), 2.46 (*d*, *J*=0.6 Hz, 3H, $C\underline{H}_3$-isoxazole), 2.86 (*d*, *J*=5.1 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.28 (*d*, *J*=5.1 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.34 (*s*, 3H, $OC\underline{H}_3$), 3.41 (*dd*, *J*=6.0, 9.3 Hz, 1H, $C\underline{H}_2OCH_3$), 3.42 (*s*, 3H, $OC\underline{H}_3$), 3.64 (*dd*, *J*=7.5, 9.3 Hz, 1H, $C\underline{H}_2OCH_3$), 3.81 (*dd*, *J*=3.3, 9.3 Hz, 1H, $C\underline{H}_2OCH_3$), 3.83 (*dd*, *J*=4.5, 9.3 Hz, 1H, $C\underline{H}_2OCH_3$), 4.51 (*dq*, *J*=3.3, 6.3, 7.8, 9.6 Hz, 1H, $C\underline{H}NH$), 4.61 (*dq*, *J*=3.3, 8.7, 10.8, 12.0 Hz, 1H, $C\underline{H}NH$), 4.72 (*dt*, *J*=4.5, 7.5, 11.7 Hz, 1H, $C\underline{H}NH$), 6.39 (*t*, *J*=0.6, 1.5 Hz 1H, $\underline{H}$-isoxazole), 6.97 (*d*, *J*=8.7 Hz, 1H, $N\underline{H}$), 7.05 (*d*, *J*=7.5 Hz, 1H, $N\underline{H}$), 7.61 (*d*, *J*=6.9 Hz, 1H, $N\underline{H}$). ^{13}C-NMR (75.46 MHz, $CDCl_3$) δ: 12.58, 16.90, 21.51, 23.57, 25.33, 40.26, 50.43, 52.58, 52.87, 53.03, 59.25, 59.36, 59.58, 71.48, 71.68, 101.54, 158.26, 159.58, 169.30, 169.89, 171.63, 208.53.

(2*S*)-2-[(2*S*)-2-[(5-ethoxy-1,2-oxazol-3-yl)formamido]-3-methoxypropanamido]-3-methoxy-N-[(2*S*)-1-[(2*R*)-2-methyloxiran-2-yl]-1-oxo-3-phenylpropan-2-yl]propanamide (**54**): ^{1}H-NMR (300.05 MHz, $CDCl_3$) δ: 1.47 (*t*, *J*=6.9 Hz, 3H, $C\underline{H}_3CH_2O$), 1.49 (*s*, 3H, $C\underline{H}_3$-oxirane), 2.87 (*dd*, *J*=6.3, 13.5 Hz, 1H, $C\underline{H}_2Ph$), 2.90 (*d*, *J*=4.5 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.13 (*dd*, *J*=5.1, 14.1 Hz, 1H, $C\underline{H}_2Ph$), 3.28 (*d*, *J*=4.8 Hz, 1H, $C\underline{H}_2$ of oxirane), 3.31 (*s*, 3H, $OC\underline{H}_3$), 3.32 (*s*, 3H, $OC\underline{H}_3$), 3.38 (*dd*, *J*=6.0, 9.0 Hz, 1H, $C\underline{H}_2OCH_3$), 3.53 (*dd*, *J*=8.1, 9.3 Hz, 1H, $C\underline{H}_2OCH_3$), 3.79 (*d*, *J*=9.3 Hz, 1H, $C\underline{H}_2OCH_3$), 3.80 (*dd*, *J*=1.2, 4.2 Hz, 1H, $C\underline{H}_2OCH_3$), 4.27 (*q*, *J*=7.2, 14.4 Hz, 2H, $CH_3C\underline{H}_2O$), 4.47 (*dq*, *J*=3.3, 6.3, 8.1, 11.4 Hz, 1H, $C\underline{H}NH$), 4.67 (*dt*, *J*=4.2, 7.2, 11.4 Hz, 1H, $C\underline{H}NH$), 4.86 (*dt*, *J*=5.1, 7.5, 12.9 Hz, 1H, $C\underline{H}NH$), 5.63 (*s*, 1H, $\underline{H}$-isoxazole), 7.03 (*d*, *J*=7.2 Hz, 1H, $N\underline{H}$), 7.09 (*d*, *J*=8.1 Hz, 1H, $N\underline{H}$), 7.08-7.31 (*m*, 5H, $C_6\underline{H}_5$), 7.51 (*d*, *J*=6.9 Hz, 1H, $N\underline{H}$). ^{13}C-NMR (75.46 MHz, $CDCl_3$) δ: 14.59, 16.81, 52.67, 52.75, 52.93, 53.12, 59.32, 59.46, 59.51, 69.23, 71.34, 71.47, 127.24, 128.69, 129.73, 135.96, 159.49, 159.69, 169.70, 174.78, 207.26.

Table S1. Solubility data of compounds **11**, **28**, **29**, **46**, **49** and **58**[a]

Compd.	Solubility [b] (mg/mL)
11	0.06 (0.05, 0.06)
28	1.48 (1.45, 1.50)
29	0.75[c] (0.66, 0.84)
46	1.24 (1.00, 1.48)
49	5.88 (5.36, 6.40)
58	1.86 (1.83, 1.89)

[a] Solubility in 10% (v/v) EtOH, 10% (v/v) PS80 citrate buffer pH=3.5; [b] solubility data averaged from two independent runs and individual data listed in parenthesis.; [c] formulations for animal dosing were prepared by first dissolving analog in EtOH and then diluting with 10% (v/v) PS80 and pH=3.5 aqueous solution, therefore concentration >1.0 mg/mL were achieved.

Table S2. Statistical analysis of anti-tumor response of compounds **2, 54** and **58**

Efficacy studies	Group	Time to achieve statistical significance [a] (days)	Statistical significance at the end of study [a, b] (P value)
RL model	**2** i.v. 5 mg/kg	35 (P<0.05)	<0.01
	54 p.o. 20 mg/kg	NA[c]	NA[c]
	58 p.o. 30 mg/kg	26 (P<0.05)	<0.001
CT-26 model	**2** i.v. 5 mg/kg	26 (P<0.001)	<0.001
	54 p.o. 20 mg/kg	NA[c]	NA[c]
	58 p.o. 30 mg/kg	26 (P<0.01)	<0.01

[a] Comparison to vehicle; [b] RL model: 38-day study and CT-26 model: 26-day study; [c] not achieved.

Table S3. Potency of key analogs for inhibition of the chymotrypsin-like activity of the 26S Proteasome[a]

Compd.	k_{inact}/K_i (M^{-1} s^{-1})
2	107,700 ±16,700
5	7,900 ±1,900
11	50,900 ±16,400
28	28,300± 6,700
29	58,600 ±10,800
49	31,800± 1,600
58	9,300± 900

[a] LLVY-amc (10µM) hydrolysis by the 26S proteasome (3nM) in buffer (20mM TRIS pH 8.0, 1mM $MgCl_2$, 1mM DTT, 0.5mM) was monitored spectrophotometrically and k_{inact}/K_i values were determined as previously described in reference 22.

Table S4. Purity of key analogs determined by aforementioned LCMS method

Compd.	Retention time (minutes)	Purity (%)	LRMS ($M+H^+$)	Calculated M.W.	Molecular Formula
5	7.7	95	559.85	558.71	$C_{30}H_{46}N_4O_6$
11	16.6	95	541.78	540.65	$C_{29}H_{40}N_4O_6$
28	13.1	96	626.77	625.76	$C_{33}H_{47}N_5O_7$
29	13.4	97	529.67	528.60	$C_{27}H_{36}N_4O_7$
46	8.1	95	453.67	452.50	$C_{21}H_{32}N_4O_7$
49	9.2	95	483.62	482.53	$C_{22}H_{34}N_4O_8$
54	11.0	98	547.73	546.57	$C_{26}H_{34}N_4O_9$
58	7.2	95	533.59	532.61	$C_{25}H_{32}N_4O_7S$