Non-competitive modulation of the proteasome by imidazoline scaffolds overcome bortezomib resistant and delay MM tumor growth in vivo

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SUPPLEMENTAL

Supplemental Methods

Determination of critical micelle concentration and drug aggregation

A method based on the tautomerization of benzoylactone was used to determine the critical micellar concentration of TCH-013.[1, 2] The Critical Micelle Concentration was determined to be the amount of agent necessary to shift the band centered at λ250 (due to the keto form of benzoylacteone) to the band centered at λ312 (due to the enolic form of benzyolacetone) as measured on a SpectraMax M5e spectrometer.

To determine aggregate formation, the fluorogenic substrate Suc-LLVY-AMC was used to measure CT-L proteasome activity pre- and post-centrifugation. Reactions (1 mL) were set up in 1.5 mL microcentrifuge tubes and contained 1 nM of purified human 20S proteasome in 50 mM Tris-HCl pH 7.5 and 0.03% SDS with either vehicle or concentrations of TCH-013 ranging from

1-20 μM. CT-L activity was measured as above, using 200 μL aliquots of the pre-centrifuged reaction mix transferred to a black clear bottom 96-well plate after adding 50 μM fluorogenic Suc-LLVY-AMC substrate. The remainder of the reaction mix was centrifuged at 20,000 X g for 30 minutes at 4℃. After centrifugation, 200 μL of the supernatant was aliquoted to a black clear bottom 96-well plate and CT-L activity was measured after the addition of 50 μM fluorogenic CT-L substrate as described above. The IC_{50} values were compared from pre-centrifuged and post-centrifuged samples to determine if TCH-013 aggregated. In the determination of aggregate formation, protealytic assays were also performed in assay buffer with the addition of either 0.5% BSA, 0.1% trypsin or 0.04% triton X-100.

Bioavailability of TCH-013

To assess the bioavailability of TCH-013 administered via intraperitoneal injection (i.p.), female immune competent BALB/c mice (6-8 weeks of age) were weighed and distributed into treatment groups (n=5). Mice received 150 mg/kg TCH-013 in 10% propylene glycol/water) via intraperitoneal injection. One, three and six hours post drug treatment blood was drawn via saphenous vein collection into heparinized 75 mm hematocrit tubes (Drummond). Isolated serum (10 μL) was added to 200 μL of ice cold HPLC grade acetonitrile in a microcentrifuge tube. The samples were vortexed vigorously for 10 minutes at 4℃ and centrifuged at 5000g for 15 minutes at 4^0 C. After centrifugation, 100 μL of the supernatant was transferred to a 96-well polypropylene microtiter plate and analyzed for TCH-013a concentration levels by LC/MS on a Waters LCT Premier mass spectrometer equipped with Shimadzu SIL-5000 autosampler and LC-20AD pumps. Analysis was performed using a Waters Atlantis dC18 column (2.1 mm X 50 mm, 3 μm particles) using a flow rate of 0.3 mL/min. The gradient consisted of 10 mM aqueous

ammonium acetate (solvent A) and acetonitrile (solvent B), starting at 90% A with a linear gradient to 100% B at 5 minutes. Volumes of 10 μL were injected and analyzed using positive mode electrospray ionization. Retention times and response factors were obtained through analysis of authentic standards of TCH-013.

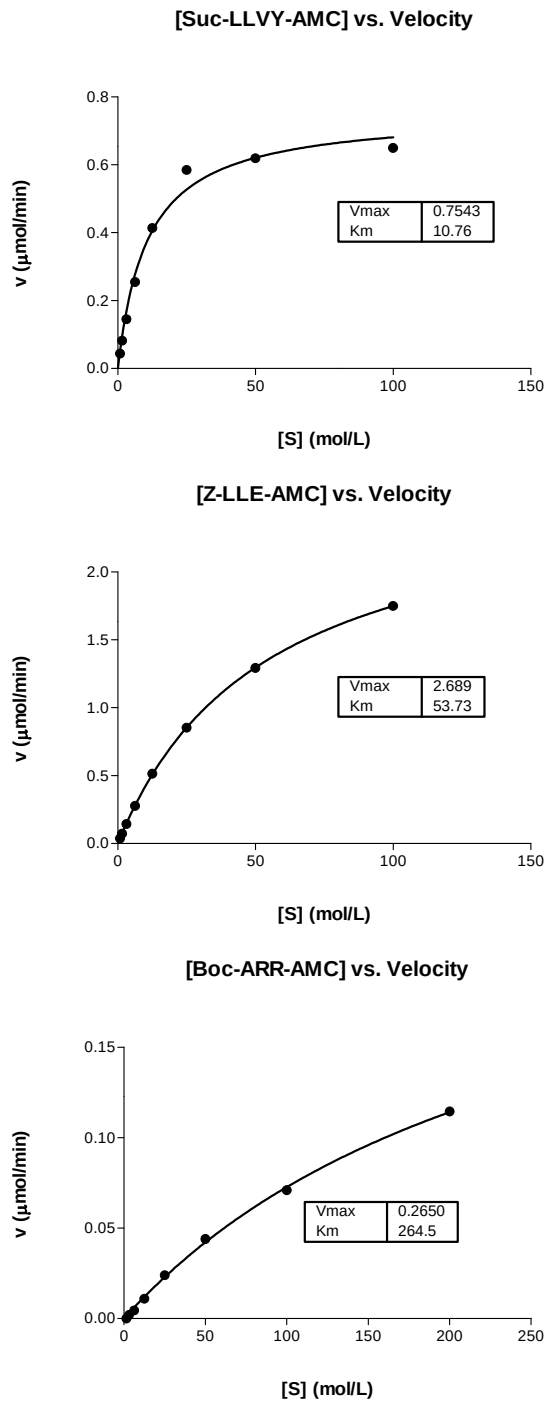
Reversibility of TCH-013

Reversibility of proteasome inhibition with TCH-013 was tested using a membrane washout assay. The CT-L activity with 5 μM Suc-LLVY-AMC was measured in the following samples: (1) 1 nM human proteasome (vehicle added), and then washed with 500 volumes of 50 mM Tris/HCl buffer, pH 8, on a membrane concentrator (Amicon Ultracel regenerated cellulose centrifugal filter, 10,000 MWCO); (2) proteasomes pretreated for 10 min with 10 μM TCH-013 and then washed as above; and (3) proteasomes pretreated with 1 μM MG-132 and washed as above.

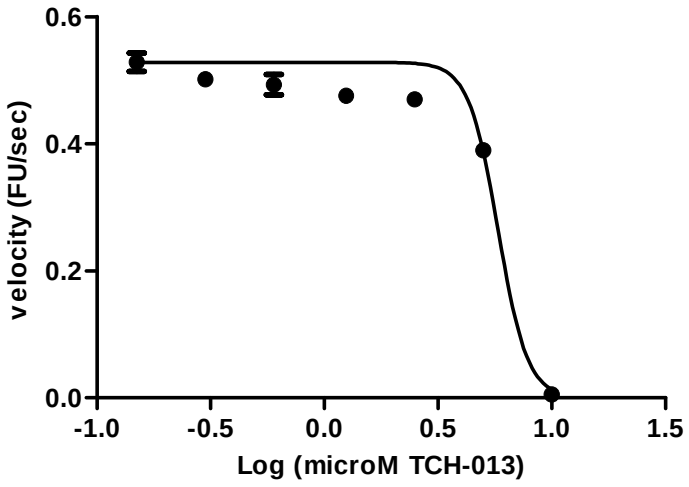
Human MM xenograft model

All procedures carried out in this experiment were conducted in compliance with all the laws, regulations and guidelines of the National Institute of Health (NIH) and with the approval of Discovery and Imaging Services, Ann Arbor's DIS-AA Animal Care and Use Committee. DIS-AA is an AAALAC accredited facility. All mice were observed for clinical signs at least once daily. All mice were dosed according to individual body weight on the day of treatment (0.2 mL/20g). Body weights and tumor measurements were recorded five times weekly. Mice were weighed on each day of treatment and at least twice weekly thereafter. Treatments began on day 34, when the mean estimated tumor mass for all groups in the experiment was 122 mg (range of group means, 122-124 mg). All mice weighed ≥20.2 g at the initiation of therapy. Mean group

body weights at first treatment were well matched (range of group means, 20.9-21.9 g). Bortezomib was dissolved in normal saline (0.9% NaCl) on each day of administration. The formulation was sonicated for 5 minutes to create a clear solution with a pH of 5.5. TCH-013 was stored at 4℃. Dosing solutions for TCH-013 were prepared on each treatment day. The compound was dissolved in pre-warmed propylene glycol, 10% of total vehicle volume and sterile water, prewarmed to 37℃ was added with vortexing so that the final vehicle contained 10% propylene glycol and 90% sterile water. This produced a clear solution with a pH of 3.5.



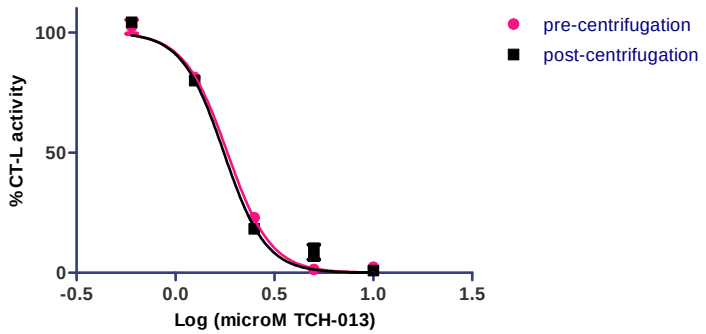
Supplemental Fig. 1. **Determination of K_m for substrates Suc-LLVY-AMC, Z-LLE-AMC and Boc-ARR-AMC**. Human purified 20S particles (1 nM) was activated in 0.03% SDS, 10 mM Tris-HCL pH 7.5 and substrate was added in a range of 0-200 µM. The maximum increase in fluorescence per minute was used to calculate specific activities of each sample and K_m was determined by Michaelis-Menton analysis.



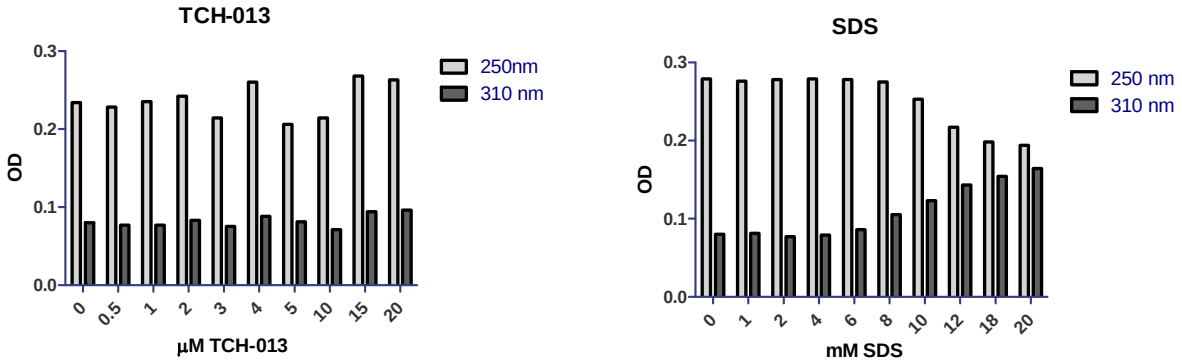
	TCH-013
log(inhibitor) vs. response -- Variable slope (four parameters)	
Best-fit values	
Bottom	= 0.0
Top	= 0.5280
LogIC50	0.7646
HillSlope	-6.748
IC50	5.816
Span	= 0.5280
Std. Error	
LogIC50	0.03169
HillSlope	2.982

Supplemental Fig. 2. Dose response of *S. cerevisiae* 20S proteasome to TCH-013. 20S proteasome purified from *S. cerevisiae* (1 nM) was activated in 0.03% SDS, 10 mM Tris-HCl pH 7.5 and 5 μM Suc-LLVY-AMC substrate was used to measure the CT-L activity according to materials and methods. The data was fit to a four parameter variable slope to determine the IC_{50}.

A



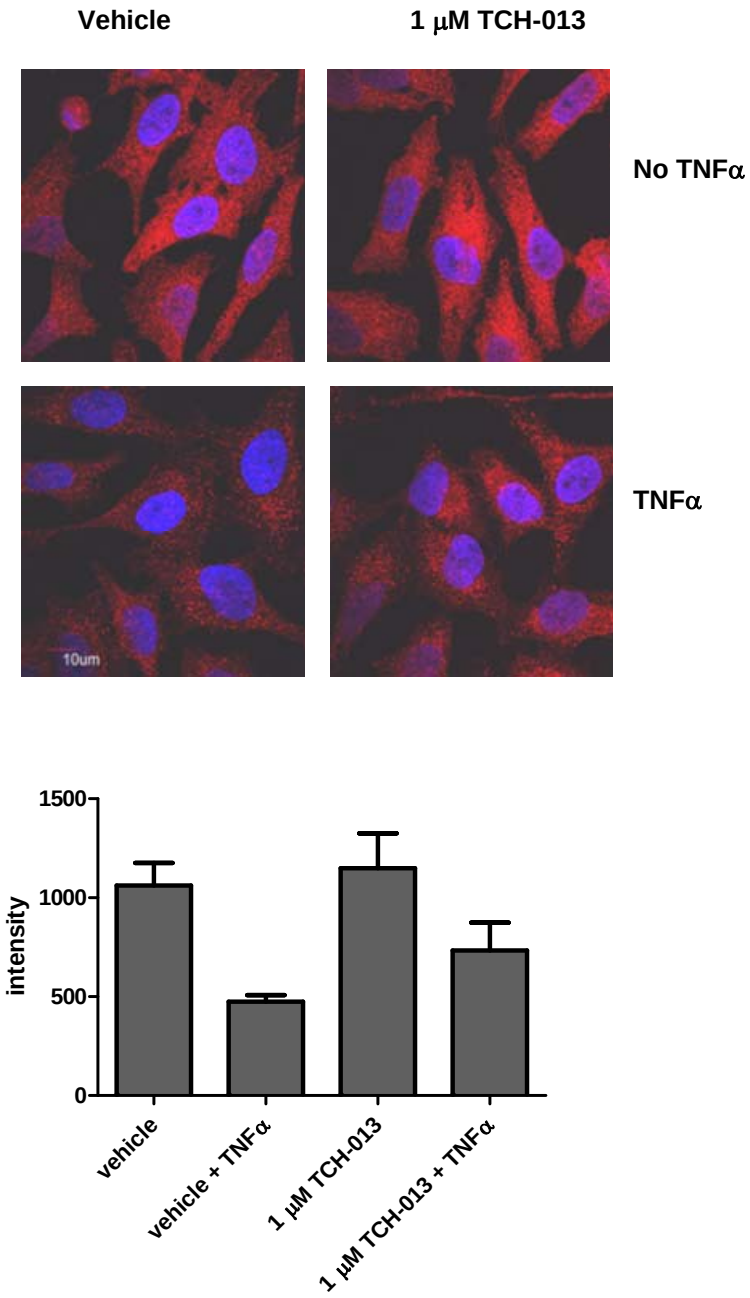
B



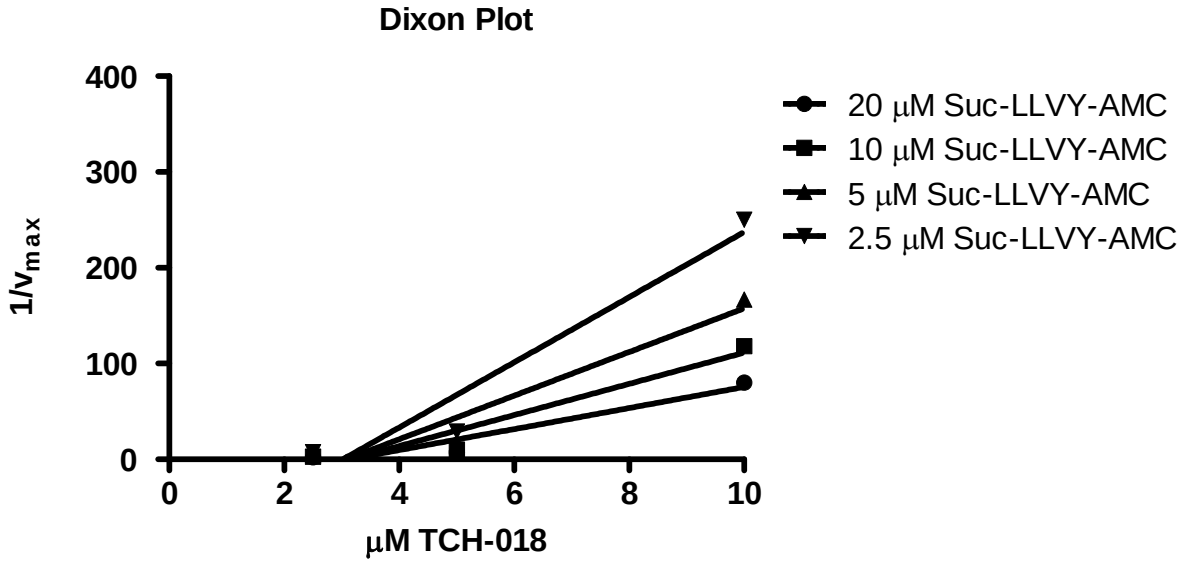
Supplemental Fig. 3. TCH-013 does not form colloidal aggregates with the 20S proteasome. **(A)** The IC_{50} of TCH-013 does not change after high speed centrifugation of the reaction. Purified human 20S proteasome (1 nM) was activated with 0.03% SDS, 10 mM Tris-HCl and treated with TCH-013. The CT-L activity of the proteasome was measured as described in materials and methods. The reaction was centrifuged at 15,000 X g at 4˚C for thirty minutes and the CT-L activity of the proteasome was measured again. The data was fit to a four parameter variable slope to determine the respective IC_{50} values. **(B)** TCH-013 does not reach a critical micellar concentration (CMC) at doses concentrations (tested up to 20 µM). CMC was determined based on the tautomerization of benzoylacetone described in the supplemental methods. Purified human 20S proteasome was activated with 0.3% SDS, 10 mM Tris-HCl, pH 7.5 and treated with either increasing amounts of SDS or TCH-013. While micelle formation of SDS (at about 8 µM) induces a shift of the band centered at λ250 to the band centered at λ310 due to the keto form of benzoylacetone tautomerizing to the enolic form of benzoylacetone, there appears to be no shift with the addition of TCH-013 up to 20 µM.

	IC50 (μM)	Concentration tested (μM)	% CT-L Activity			
			No competitor	0.5% BSA	0.1% Trypsin	0.04% triton X-100
TCH-013	2.5	10	0%	0.00%	0%	58.50%
bortezomib	0.008	0.05	0%	0.00%	0%	3.50%

Supplemental Table 1. TCH-013 does not inhibit the proteasome through aggregate formation. The IC_{50} of TCH-013 and bortezomib was determined using 1 nM purified human 20S proteasome activated with 0.3%SDS, 10 mM Tris-HCl pH 7.5 with 5 μM Suc-LLVY-AMC according to the procedure in materials and methods. The percentage of CT-L activity was determined with the addition of no competitor, 0.5% BSA, 0.1% trypsin or 0.04% triton X-100 before the addition of TCH-013.



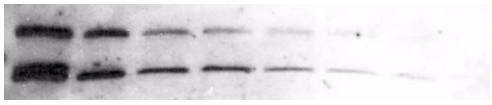
Supplemental Fig. 4. Confocal Microscopy of IκBα stabilization after TCH-013 treatment using antibody directed toward the N-terminus of IκBα.). Representative photos showing fluorescent confocal images of HeLa cells treated with either vehicle or 1 μM TCH-013 and either stimulated with 10 ng/mL TNF-α or left untreated. The cells were fixed and subsequently stained for the presence of IκBα (red) and counterstained for DNA with DAPI (blue).



[I]	Vmax	Vmax (app)	Ki
0	0.991	0.99	--
5	0.991	0.24	2.2
2.5	0.991	0.72	2.8
1.25	0.991	0.85	2.1
		mean	**2.4**
		stdev	**0.4**

Supplemental Fig. 5. Determination of K_i of TCH-013 using for substrates Suc-LLVY-AMC using Dixon plot. Human purified 20S particles (1 nM) was activated in 0.03% SDS, 10 mM Tris-HCL pH 7.5 and substrate was added in a range of 0-20 μM. The maximum increase in fluorescence per minute was used to calculate specific activities of each sample and K_i was calculated based on the data obtained from the Michaelis-Menton derived Vmax and apparent Vmax using the following equation: $Vmax_{app} = Vmax/(1+[I]/Ki)$.

A



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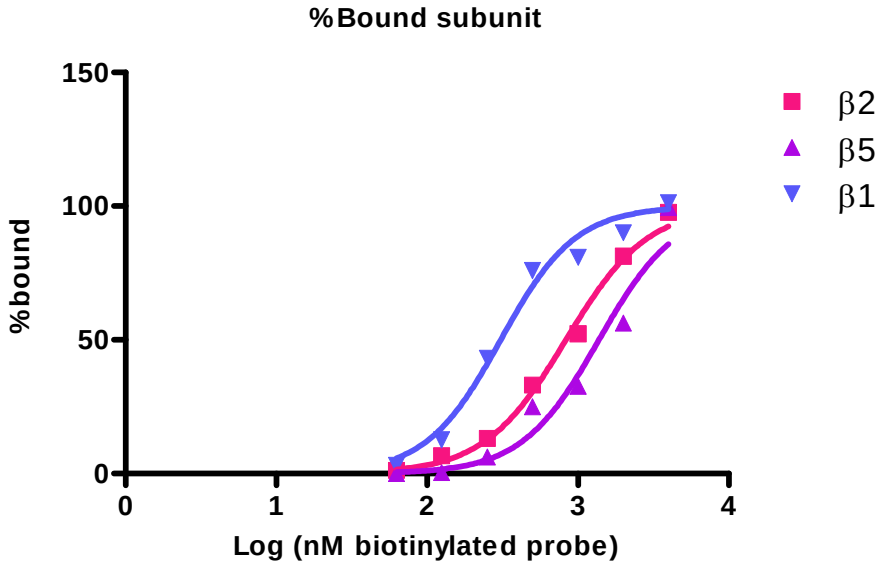
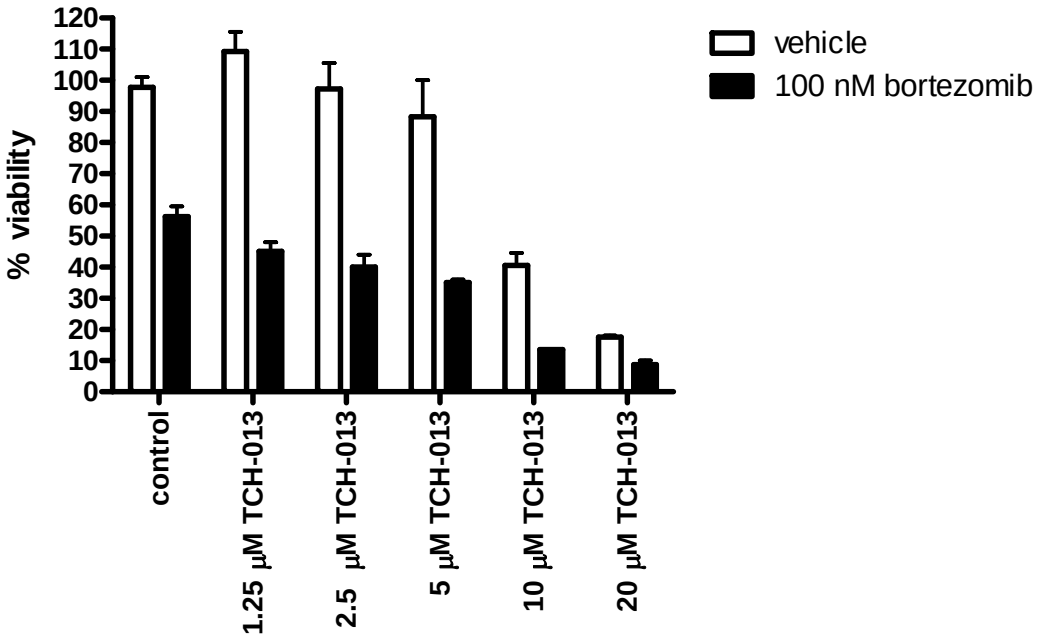
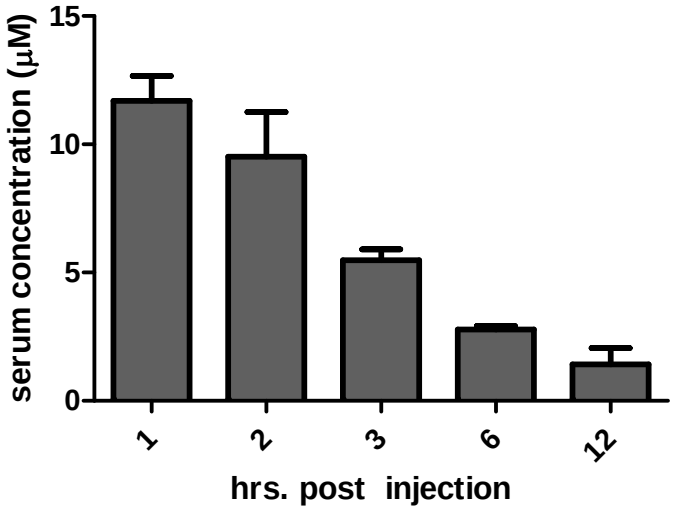


	T-L	CT-L	PGPH
Sigmoidal dose-response (variable slope)			
Best-fit values			
Bottom	= 0.0	= 0.0	= 0.0
Top	= 100.0	= 100.0	= 100.0
LogEC50	2.921	3.139	2.488
HillSlope	1.599	1.693	1.752
EC50	832.7	1378	307.6
Std. Error			
LogEC50	0.02537	0.05999	0.03939
HillSlope	0.1351	0.3706	0.2492

Supplemental Fig. 6. **A**. Western blot of the biotinylated probe Ada-Lys(biotinyl)-$(Ahx)_3$-$(Leu)_3$-vinyl sulfone at various concentrations. **B**. Determination of half maximal binding of the biotinylated probe Ada-Lys(biotinyl)-$(Ahx)_3$-$(Leu)_3$-vinyl sulfone.

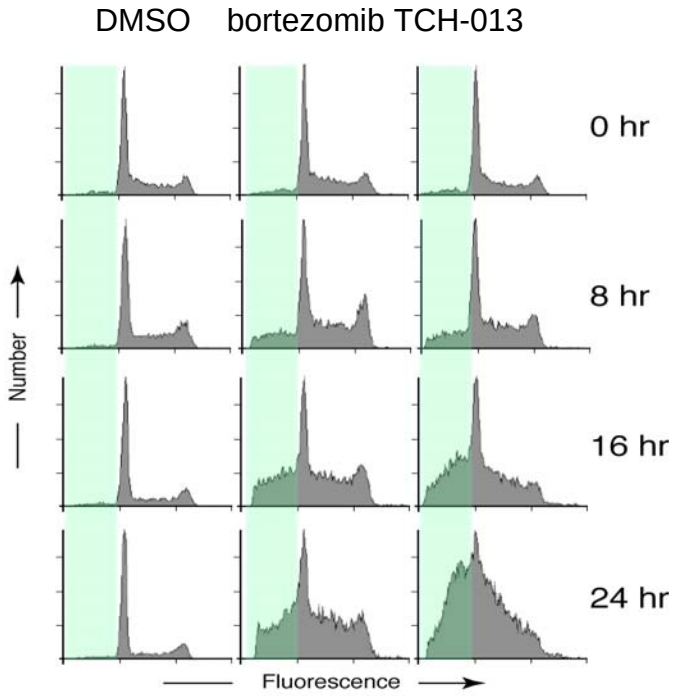


Supplemental Fig. 7. L363 Cells treated with TCH-013 or combination of TCH-013/100 nM bortezomib for 15 hours and viability measured by MTS

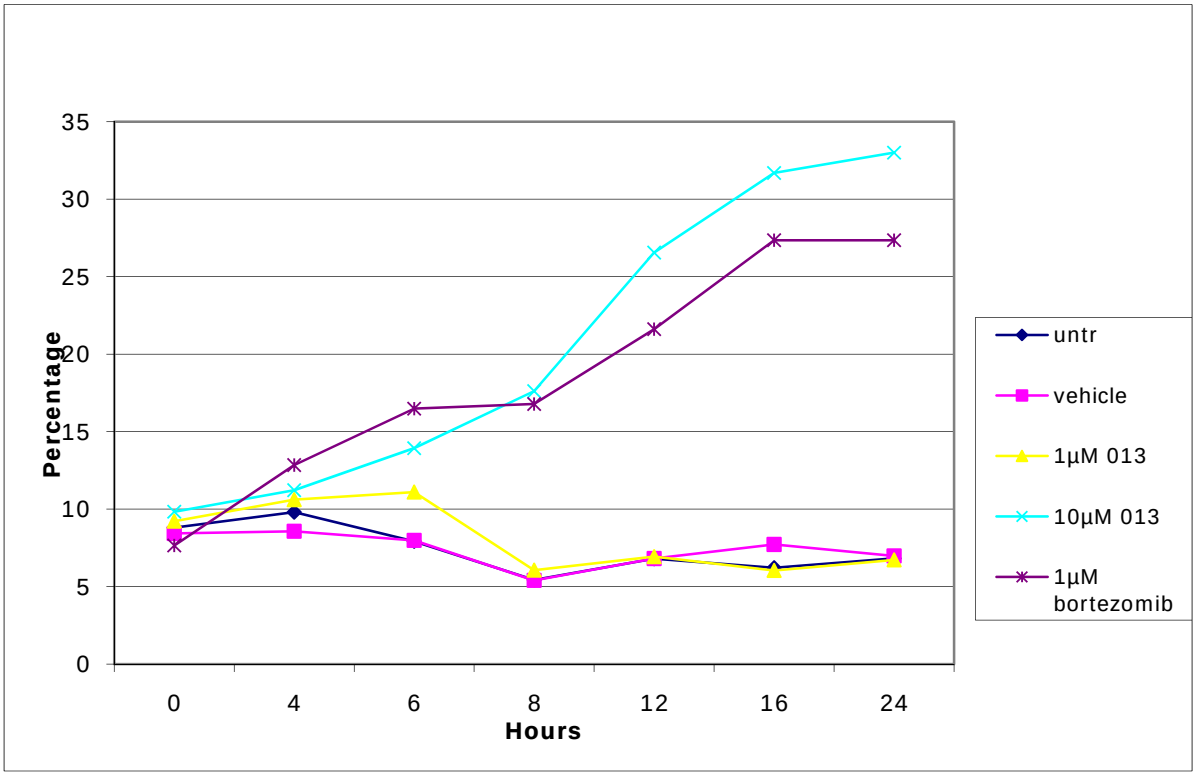


Supplemental Fig. 8. Blood serum levels of TCH-013 in BALB/c mice. Blood levels of TCH-013 were measured by LC/MS at 1, 2, 3, 6 and 12 hours post IP injection of 150 mg/kg TCH-013. (K_{elim} = 0.203 ± 0.003, $T_{1/2}$ = 3.3 ± 0.2 hours)

A

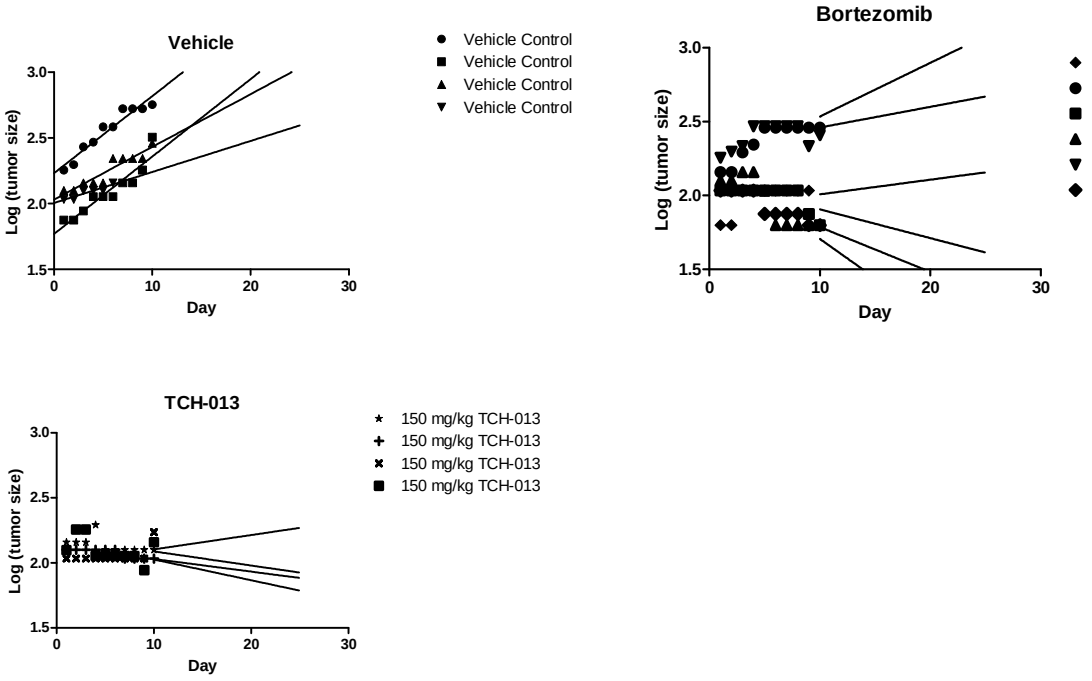

DMSO
bortezomib
TCH-013
0 hr
8 hr
16 hr
24 hr
Number
Fluorescence

B


35
30
25
20
15
10
5
0
Percentage
0
4
6
8
12
16
24
Hours
untr
vehicle
1µM 013
10µM 013
1µM bortezomib

	0 hr.	4 hr.	6 hr.	8 hr.	12 hr.	16 hr.	24 hr.
untreated	8.80	9.80	7.92	5.43	6.80	6.21	6.82
vehicle	8.43	8.57	7.98	5.40	6.81	7.72	6.98
1μM 013	9.22	10.61	11.10	6.06	6.93	6.04	6.72
10μM 013	9.83	11.22	13.93	17.61	26.55	31.69	33.00
1μM bortezomib	7.65	12.84	16.48	16.78	21.61	27.35	27.35

Supplemental Fig. 9(A) TCH-013 causes a rapid increase in DNA fragmentation in RPMI-8226 cells, which is indicative of apoptosis. Human MM cell line RPMI-8226 cells were treated with either: vehicle, bortezomib, or TCH-013 for 0, 8, 16 and 24 hours. The cells were stained with propidium iodide and the cells were analyzed by fluorescence activated cell sorting to measure cellular chromosomal DNA content. (**B)** Percentage of RPMI-8226 cells in sub-G1.



	y-intercept	x-intercept	slope
Vehicle			
M1	2.231	-38.100	0.058
M2	1.769	-30.000	0.058
M3	2.031	-50.700	0.040
M4	2.005	-85.000	0.023
Bortezomib			
M1	1.909	-192.200	0.009
M2	2.171	-59.700	0.036
M3	2.101	107.800	-0.019
M4	2.237	42.160	-0.053
M5	2.320	-165.700	0.014
M6	2.090	68.800	-0.030
TCH-013			
M1	2.197	202.100	-0.010
M2	2.127	218.400	-0.009
M3	1.993	-180.800	0.011
M4	2.185	136.900	-0.015

Supplemental Fig 10. Log-linear regression analyses of individual mice during the time of treatment.

References:

1. Dominquez, A.; Fernandez, A.; Gonzalez, N.; Iglesias, E.; Montenegro, L., Determination of critical micelle concentration of some surfactants by three techniques. *J. Chem. Ed.* **1997,** 74, 1227-1231.
2. Lansdell, T. A.; Hewlett, N. M.; Skoumbourdis, A. P.; Fodor, M. D.; Seiple, I. B.; Su, S.; Baran, P. S.; Feldman, K. S.; Tepe, J. J., Palau'amine and Related Oroidin Alkaloids Dibromophakellin and Dibromophakellstatin Inhibit the Human 20S Proteasome. *J. Nat. Prod.* **2012,** 75, (5), 980-5.