Supplemental Materials and Methods

Antibodies and Reagents

Dexamethasone sodium phosphate was obtained from the VU University Medical Center pharmacy. Bortezomib was kindly provided by Millennium Pharmaceuticals (Cambridge, MA, USA). 5-Amino-8-Hydroxyquinole (5AHQ) was synthesized by Dr A.D. Schimmer (Toronto, Canada)[1]. The epoxyketone-based proteasome inhibitors carfilzomib, ONX 0912 and ONX 0914 were from Onyx Pharmaceuticals (South San Francisco, CA, USA). All drugs were prepared as 10 mM stock solutions in DMSO, aliquoted for single use and stored at -80°C. β-actin (clone c4) antibody was obtained from Boehringer Mannheim (Almere, The Netherlands). Antibodies to proteasome subunits β1, β2, β5, β1i, β5i, and α7 were from Enzo Life Sciences (Farmingdale, NY, USA).

Cell culture

Human monocytic/macrophage THP1 cells and T-ALL CCRF-CEM cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium containing 2 mM glutamine (Invitrogen/Gibco (Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) and 100 U/ml penicillin/streptomycin (Invitrogen) at 5% CO_2 and 37 °C. Cell cultures were seeded at a density of $3x10^5$ cells/ml and refreshed twice weekly.

MTT cytotoxicity assay

For drug combination studies in patient samples, ALL ($2x10^6$ cells/ml), or AML cells ($1x10^6$ cells/ml) were incubated with 5 different fixed concentrations of single drug bortezomib or dexamethasone and their combination in 150 µl medium in a 96-wells plate for 96 hours at 37^0C. To determine which concentrations of dexamethasone and bortezomib were optimal for drug combination assays, 8 patient samples were initially tested for a wide range of concentrations. For single-drug dexamethasone, an 8-fold dilution range of 6 µM – 0.18 nM was tested. For single-drug bortezomib, a 1.7-fold-dilution range of 285 nM - 0.83 nM was used. Based on these settings, a non-constant ratio combination design was used for combination studies, in which 2-4 different concentrations of bortezomib were combined with a 5-fold-dilution range of dexamethasone (750 nM – 0.18 nM). Cells were pre-

incubated with freshly prepared bortezomib for 2 hours at 37^0C and then added to a 96-wells plate containing different dexamethasone concentrations. After 96 hours of incubation at 37^0C, 15 µl of MTT (5 mg/ml) was added to the wells. Plates were then incubated for another 6 hours at 37^0C and the formed formazan crystals were solubilized by mixing prior to spectrophotometric determination at 540 and 720 nm using the Anthos 2001 microplate spectrophotometer (Anthoslabtec B.V. Heerhugowaard, the Netherlands). Results are presented as the lethal concentrations that result in 50% cell kill when compared to untreated controls (LC_{50}). It has to be emphasized that these primary patient samples do not proliferate in this assay and the cytotoxicity cannot be attributed to growth inhibition.

In vitro drug sensitivity was determined using the 4-day MTT cytotoxicity assay as described previously[2]. For THP1, bortezomib (range 0.0014 µM – 0.033 µM) and ONX 0914 (range: 0.0005 µM – 1 µM) were added to the plate for 4 days. CCRF-CEM cells were pulse-treated with or without 1 µM bortezomib for 1h, followed by two washing steps before single drugs and combinations of Bortezomib (range 0.0005 µM – 0.0056 µM) and dexamethasone (range: 0.0056 µM – 0.031 µM) were added to the plate for 4 days. The IC_{50} value was defined as the drug concentration needed to inhibit 50% of the cell growth compared to growth of the untreated control cells.

Analysis of drug effects

Leukemic cell survival after MTT assays was calculated as follows: the optical density (OD) of the treated well (-blank) / mean OD of the control well (-blank) x 100. Mutually non-exclusive CIs were used for experimental values, because dexamethasone and bortezomib have different modes of action. These equations were used to calculate synergistic, additive, and antagonistic drug interactions. A CI in the range of 0.9 and 1.1 is considered to be an additive effect. Whereas, CI values < 0.9 and CI > 1.1 indicate synergistic and antagonistic effects, respectively.

RNA Interference

For RNA interference experiments all targeted and non-targeted siRNA constructs were obtained from Dharmacon (Lafayette, USA). THP1 cells were cultured following the

DharmaFECT general transfection protocol conditions for THP1 cells. Briefly, prior to transfection, cells were cultured overnight at a density of 0.3×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS. Cells were transfected using Dharmafect 2 and 100 nM of *PSMB8* or *PSMB9* On-Targetplus SmartPool siRNA. As negative control, 100 nM On-targetplus siControl non-targeting was included.

ProCISE analysis

A previously described[3] ELISA-based method (proCISE) was used to quantify the fraction of constitutive and immunoproteasome subunits per patient. Briefly, the method quantifies the amount of each subunit using luminescence assay and then this measure is translated into ng of proteasome or µg/ml of lysate by comparison with the 20S proteasome standard curve. The lower limit of detection (LLoD) for this method was calculated for each standard curve. Here LLoD was defined using a set of control samples with known concentrations. The LLoD threshold was set by finding the first control sample to show a deviation of >0.25 from the known concentration and <3 standard deviations from the blank control.

For samples within the dataset with levels below LLoD (BLLoD) concentrations of constitutive proteasome subunits, we utilized statistical methods that account for this type of censored data in our analysis. To analyze the censored data the package NADA in R (version 2.15) was used. The Regression on Ordered Statistics (ROS) method was used to impute the subunit concentrations for those samples that fell below the LLoD threshold. These imputed values were then treated as non-censored values in further analysis. The concentration values estimated from the standard curve were normalized for each sample by the measured concentration of total protein per sample to account for differences due to input amount.

To assess the difference in total proteasome, constitutive proteasome and immunoproteasome between the ALL and AML samples, we used a Mann-Whitney U test. The Mann-Whitney U test was also used to assess the difference between these disease groups when looking at the immunoproteasome/constitutive proteasome subunits values. Both of these analyses were carried out in R (v2.15).

cDNA synthesis and quantitative RT-PCR

After RNA isolation by the RNAeasy mini kit (Qiagen, Valencia, CA, USA), cDNA was synthesized using RT buffer (Invitrogen), containing 5 mM DTT (Invitrogen), 2 mM dNTP (Roche), pdN6 96 ug/ml (Roche), 0.75 U/ul M-MLV (Invitrogen) and 2 U/ul RNAsin (HT Biotechnology Ltd., Cambridge, UK). mRNA expression levels of proteasome subunits *PSMB8, PSMB9* and GUS as a reference were quantified using real-time PCR analysis (Taqman) on an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). All primers and probes were designed using Primer Express software (Applied Biosystems). Probes were labeled with 5'-FAM and 3'-BHQ1 as a reporter. Real-time PCR was performed in a total reaction volume of 25 µl containing TaqMan buffer A (Applied Biosystems), 4 mM $MgCl_2$, 0.25 mM of each dNTP (Amersham Pharmacia Biotech) and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Samples were heated for 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase and amplified during 40 cycles of 15 s at 95°C and 60 s at 60°C. Relative mRNA expression levels of the target genes in each sample were calculated using the comparative cycle time (Ct) method. The Ct of the target gene is normalized to the GUS Ct value by subtracting the GUS Ct value from the target Ct value. The mRNA expression level for each target PCR relative to GUS was calculated using the following equation: mRNA expression = $2^{(\text{Ct target-Ct GUS})} \times 100\%$.

References

[1] Li X, Wood TE, Sprangers R, Jansen G, Franke NE, Mao X, et al. Effect of noncompetitive proteasome inhibition on bortezomib resistance. *J.Natl.Cancer Inst.* 2010;102(14):1069–82.

[2]Van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. *Methods Mol.Biol.* 2011;731:237–45.

[3] Suzuki E, Demo S, Deu E, Keats J, Rastu-Kapur S, Bergsagel PL, et al. Molecular mechanisms of bortezomib resistant adenocarcinoma cells. *PLoS.One.* 2011;6(12):e27996.

Supplemental tables

Table S1. Proteasome subunit expression and ex vivo sensitivity to proteasome inhibitors and of pediatric AML and ALL patient samples

	ALL			AML			
Pro-CISE	N	Median expression ng/µg total protein	Range	N	Median expression ng/µg total protein	Range	*P*
β5	19	1.27	0.6 - 3.04	6	0.71	0.44 - 1.66	0.176
β5i	19	6.66	3.34 - 9.81	6	6.16	3.02 - 7.46	0.274
β1	19	1.46	0.55 - 2.69	6	2.24	1.16 - 3.53	0.156
β1i	19	6.21	2.46 - 11.4	6	4.10	1.28 - 6.87	0.05
β2	19	3.13	1.91 - 6.77	6	3.03	2.26 - 4.41	1.00
β2i	19	5.62	1.65 - 10.3	6	2.94	2.46 - 4.66	0.03
Total proteasome	19	25.9	15.2 - 39.6	6	19.9	12.8 - 23.7	0.05
Immunoproteasome	19	18.4	8.22 - 29.0	6	12.0	8.62 - 18.28	0.036
Constitutive proteasome	19	6.10	3.32 - 11.76	6	5.87	4.18 - 9.59	0.926
Western blotting*	N	Ratio*	Range	N	Ratio*	Range	*P*
β5	28	1.63	0.19 - 24.9	10	9.8	1.25 - 26.3	0.006
β5i	28	1.37	0.17 - 4.03	10	1.1	0.33 - 1.92	0.453
β1	29	0.05	0.00 - 0.60	10	0.24	0.13 - 0.39	0.000
β1i	29	10.9	0.90 - 20.7	10	5.7	1.21 - 11.9	0.037
β2	29	0.1	0.01 - 0.74	10	0.49	0.15 - 0.78	0.000
β2i	ND	ND	ND	ND	ND	ND	ND
α7	27	0.97	0.13 - 1.42	10	0.90	0.42 - 2.28	0.723
Drug-sensitivity	N	Median LC_{50}, nM	Range	N	Median LC_{50}, nM	Range	*P*
Bortezomib	30	6.0	3.0 – 46.1	11	14.0	10.1 – 23.4	0.000
Carfilzomib	28	4.1	0.8 - 8.7	10	20.8	6.0 - 30.8	0.000
ONX 0912	28	19.2	7.6 - 80.9	10	93.7	55.7 - 394	0.000
ONX 0914	28	44.6	8.4 - 117	10	248	89.2 - 678	0.000
5AHQ†	28	20.1†	4.9 - 122.5	10	53.8†	17.6 - 139.4	0.001
Dexamethasone	28	62.4	0.50 - >600	12	600.0	164.5 - 600	0.000

*Please note that Western Blotting data depict relative quantifications of subunit expression (ratio proteasome subunit / ß-actin based on loading of 15 ug total protein, normalized to CEM), whereas ProCISE analysis provides absolute quantification of subunits.

ND: Not Determined

† µM

Table S2. Correlations between drug-sensitivity (in LC_{50} values) and subunit ratios obtained using Pro-CISE for ALL and AML

	AML						ALL					
	β5i/β5		β1i/β1		β2i/β2		β5i/β5		β1i/β1		β2i/β2	
	Corr Coef	*P (n)*	*Corr Coef*	*P (n)*	*Corr Coef*	*P (n)*	*Corr Coef*	*P (n)*	*Corr Coef*	*P (n)*	*Corr Coef*	*P (n)*
ONX 0914	-0,371	0,468 (6)	-0,429	0,397 (6)	-0,429	0,397 (6)	-0,243	0,332 (18)	-0,527	0,025 (18)	-0,467	0.05 (18)
ONX 0912	-0,371	0,468 (6)	-0,543	0,266 (6)	-0,543	0,266 (6)	0,076	0,772 (17)	-0,150	0,567 (17)	-0,056	0,830 (17)
Carfilzomib	-0,886	0,019 (6)	-0,829	0,042 (6)	-0,829	0,042 (6)	0,167	0,523 (17)	-0,360	0,155 (17)	-0,115	0,66 (17)
Bortezomib	-0,800	0,104 (5)	-0,900	0,037 (5)	-0,900	0,037 (5)	-0,035	0,890 (18)	0,189	0,453 (18)	0,295	0,243 (18)
5AHQ	0,429	0,397 (6)	0,371	0,468 (6)	0,371	0,468 (6)	-0,269	0,280 (18)	-0,273	0,272 (18)	-0,253	0,311 (18)

Supplemental Figure legends

Figure S1. Proteasome protein subunit expression of ALL and AML patient samples determined by ProCISE. Subunit protein expression of ALL and AML patient samples is depicted as ng subunit/µg total protein of A) β5, B) β1, C) β2, D) β5i, E) β1i, and F) β2i, determined by proteasome constitutive immuno subunit ELISA. The line represents the mean.

Figure S2. Proteasome expression comparing ALL subtypes and AML. (A) Constitutive and immunoproteasome expression compared between B-ALL (n=13), T-ALL (n=4), and AML (n=6) in ng/µg total proteasome. (B) Ratios of immuno- versus constitutive subunits within B-ALL, T-ALL and AML. (C) Subdivision into pro-B ALL (n=4), pre-B ALL (n=2), common-ALL (n=7), T-ALL (n=4) and AML (n=6) compared to T-ALL cell line CCRF-CEM and AML cell line THP1. Error bars represent standard error of the mean.

Figure S3. Proteasome subunit expression of ALL subgroups. ALL patient samples divided into subgroups by immunophenotype. Protein expression of β5, β1, β2, β5i, and β1i was determined by Western blotting and normalized on actin as loading control and to the subunit expression of the CCRF-CEM cell line as control between blots. The line represents the mean.

Figure S4. Drug-sensitivity of ALL subgroups. LC_{50} concentrations of bortezomib, dexamethasone, carfilzomib, ONX 0912, ONX 0914, and 5AHQ as determined by MTT cytotoxicity assay in ALL patient samples divided into subgroups by immunophenotye. The line represents the mean.

Figure S5. *PSMB8* (β5i) silencing in THP1 cells confers diminished sensitivity to ONX 0914. (A) mRNA levels of *PSMB8* or *PSMB9* after siRNA-knockdown of *PSMB8* or *PSMB9,* respectively. (B) IC_{50} values of Bortezomib and ONX 0914 determined by 4-day MTT assays in THP1 cells after silencing *PSMB8* or *PSMB9* compared to non-target siRNA and a control without siRNA. Results depict means of 3-4 individual experiments ± S.D. *P<0.05.

Supplemental Figures

Figure S1

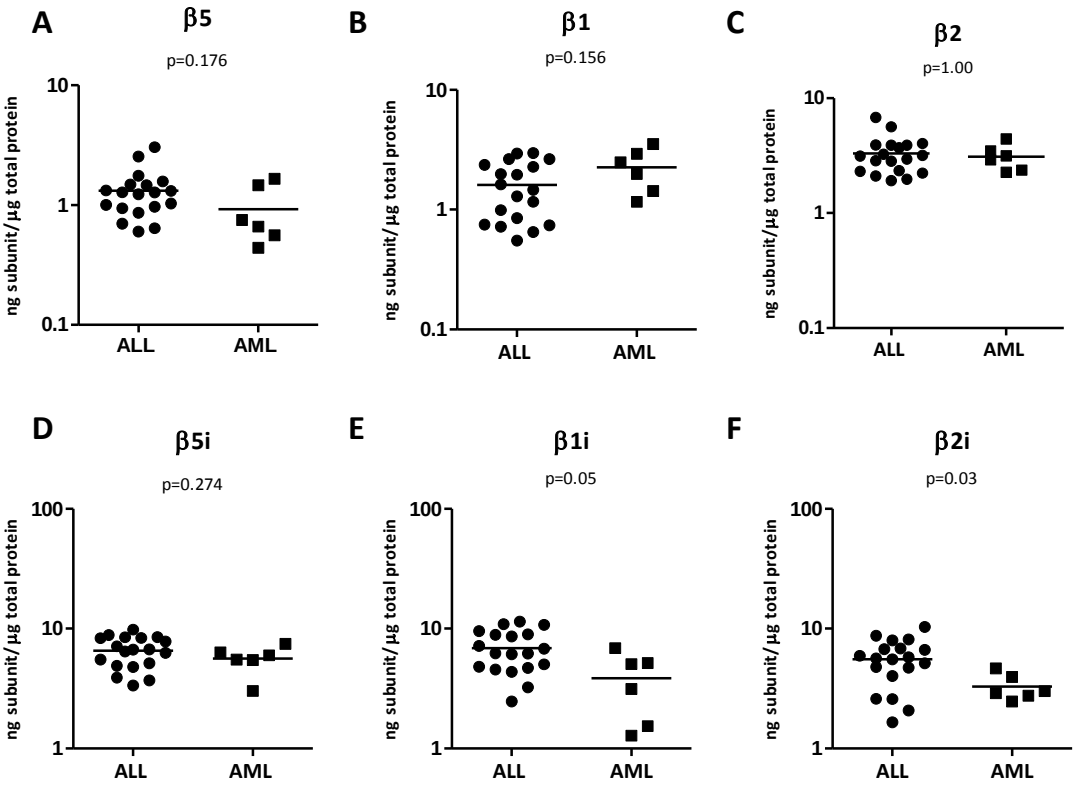


Figure S2

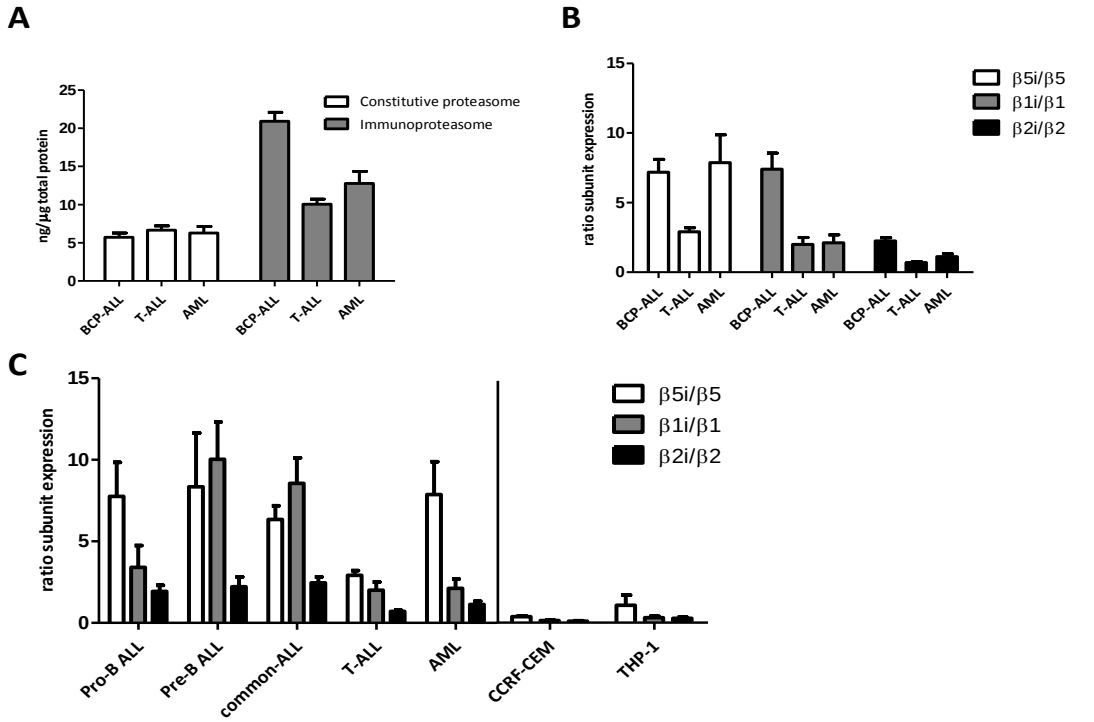


Figure S3

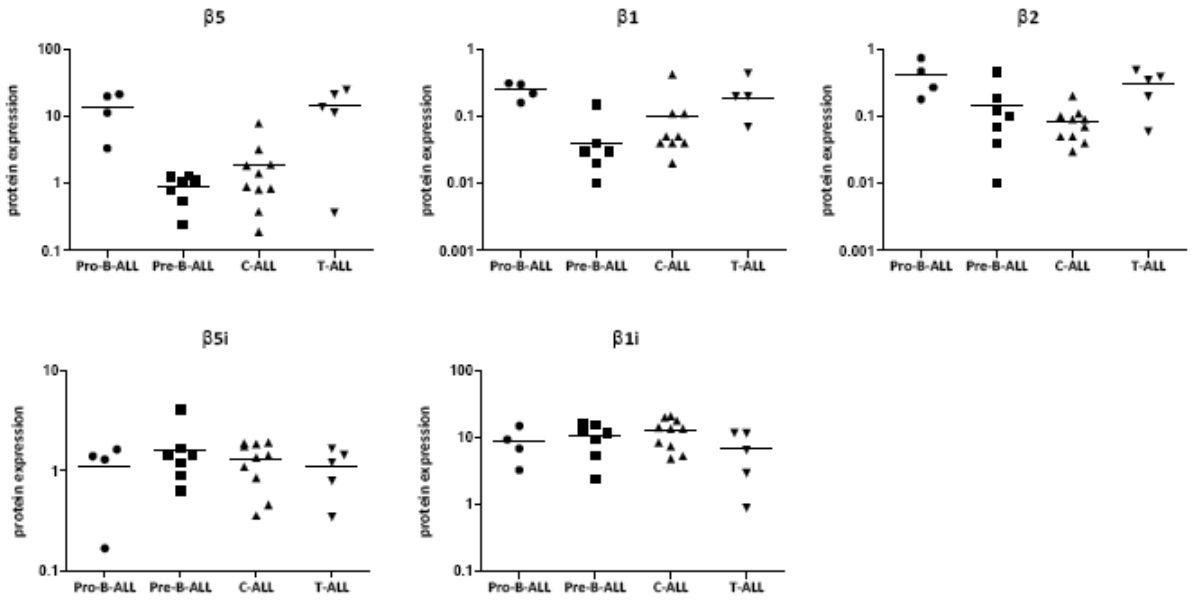


Figure S4

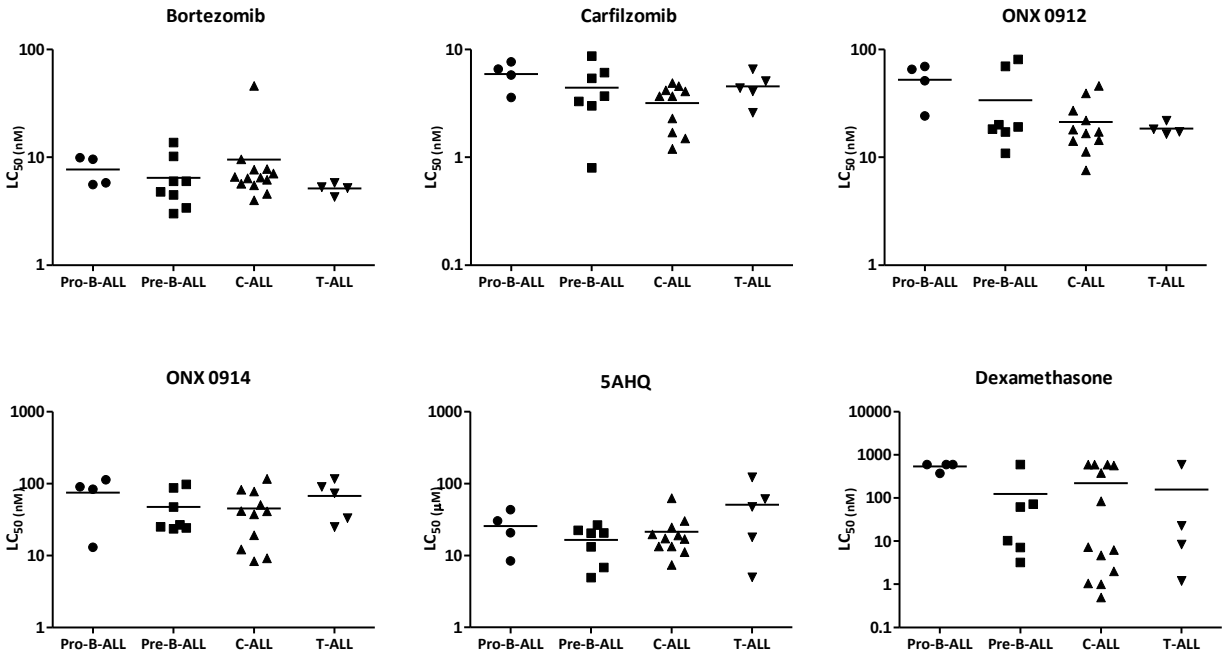
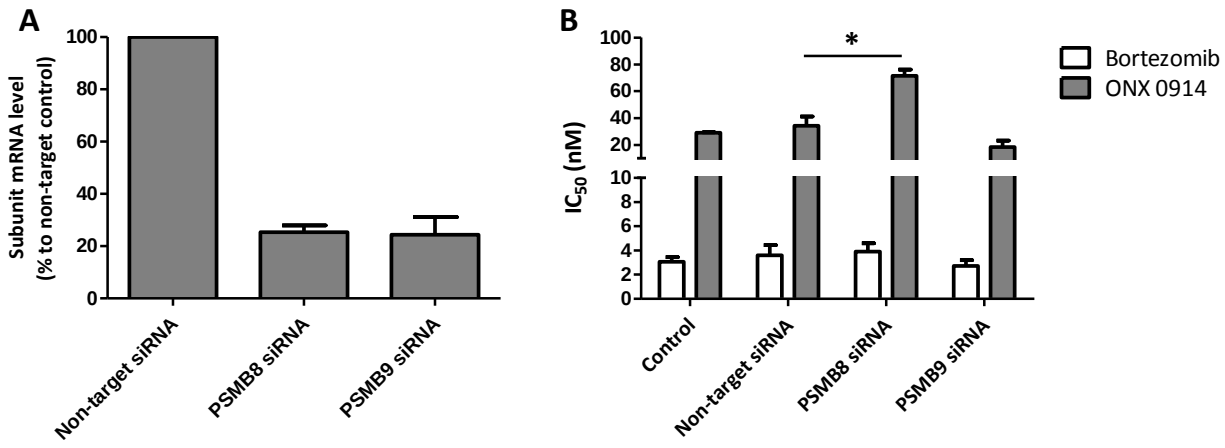


Figure S5


A
Subunit mRNA level
(% to non-target control)
100
80
60
40
20
0
Non-target siRNA
PSMB8 siRNA
PSMB9 siRNA
B
IC50 (nM)
100
80
60
40
20
10
8
6
4
2
0
*
Control
Non-target siRNA
PSMB8 siRNA
PSMB9 siRNA
Bortezomib
ONX 0914