Molecular mechanisms of bortezomib resistance in acute leukemia

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Chapter 3

Higher Ratio Immune vs. Constitutive Proteasome Level as Novel Indicator of Sensitivity of Pediatric Acute Leukemia Cells to Proteasome Inhibitors

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ABSTRACT

The *ex vivo* sensitivity of pediatric leukemia cells to the proteasome inhibitor bortezomib was compared to 3 next generation proteasome inhibitors; the epoxyketone-based irreversible proteasome inhibitors carfilzomib, its orally bio-available analogue ONX 0912, and the immunoproteasome inhibitor ONX 0914. LC_{50} values were determined by MTT cytotoxicity assays for 29 childhood acute lymphoblastic leukemia and 12 acute myeloid leukemia patient samples and correlated with protein expression levels of the constitutive proteasome subunits (β5, β1, β2) and their immunoproteasome counterparts (β5i, β1i, β2i). acute lymphoblastic leukemia cells were up to 5.5-fold more sensitive to proteasome inhibitors than acute myeloid leukemia cells ($P<0.001$) and the combination of bortezomib and dexamethasone proved additive/synergistic in the majority of patient specimens. Although total proteasome levels in acute lymphoblastic leukemia and acute myeloid leukemia cells did not differ significantly, the ratio of immuno/constitutive proteasome was markedly higher in acute lymphoblastic leukemia cells over acute myeloid leukemia cells. In both acute lymphoblastic leukemia and acute myeloid leukemia, increased ratios of β5i/ β5, β1i/ β1 and β2i/ β2 correlated with increased sensitivity to proteasome inhibitors. Together, differential expression levels of constitutive and immunoproteasomes in pediatric acute lymphoblastic leukemia and acute myeloid leukemia constitute an underlying mechanism of sensitivity to bortezomib and new generation proteasome inhibitors, which may further benefit from synergistic combination therapy with drugs including glucocorticoids.

INTRODUCTION

Recently, the crystal structures of constitutive and immunoproteasomes in the absence or presence of the novel epoxyketone-based irreversible proteasome inhibitor ONX 0914 were solved[1]. Structural evidence for selective inhibitory potential of ONX 0914 for the immunoproteasome was provided by the ability to induce conformational changes in the S1 binding pocket of the immunoproteasome subunit β5i but not in the constitutive proteasome subunit β5. Conceptually, immunoproteasome inhibitors may have dual impact on both cell proliferation and cytokine production[2,3] and could thus serve a valuable alternative to the first generation clinically active proteasome inhibitors, e.g. bortezomib, which targets both constitutive and immunoproteasome subunits[4]. Clinical evaluation of immunoproteasome inhibitors is still in an early phase, however given the established efficacy of bortezomib in the treatment of various hematologic malignancies[4,5], adult and pediatric acute leukemias are valid candidates for further (pre)clinical exploration.

Although treatment of children with acute leukemia improved throughout the last decades, 20-40% of children still relapse following initial therapy which is associated with a worse prognosis[6]. For the survival of children with acute lymphoblastic leukemia (ALL) a good initial response to glucocorticoids has favorable prognostic value[7]. Hence, glucocorticoid-resistant and relapsed ALL patients may benefit from novel and glucocorticoid-sensitizing strategies. Based on the good preclinical activity in other hematologic malignancies[5], bortezomib was selected as a novel anti-leukemic drug in pediatric leukemias[8–10]. Bortezomib is a reversible inhibitor of the 26S proteasome, a large intracellular protease expressed in all cell types[11]. The proteasome consists of seven α-subunits and seven β-subunits, three of which harbor the catalytic activities of the proteasome, chymotrypsin-like, caspase-like, and trypsin-like, encoded by β5 (PSMB5), β1 (PSMB6), and β2 (PSMB7) subunits, respectively. The catalytic activities of the proteasome are responsible for the degradation of all poly-ubiquitinated proteins[11]. Cells of the immune system express a distinct type of proteasome, the interferon-γ inducible immunoproteasome, in which all three catalytic constitutive subunits are exchanged for the immunosubunits β5i (PSMB8), β1i (PSMB9), and β2i (PSMB10)[1]. Besides immunoproteasomes, two additional proteasome hybrid types (β1+β2+β5i and β1i+β2+β5i) were identified, each of which harboring the capacity to process different tumor antigens[12]. Immunoproteasomes play a major role in the provision of peptides for antigen presentation, partly by facilitating efficient clearance of protein aggregates that arise upon interferon-induced oxidative stress[13]. Increased immunoproteasome expression has been noted in B-cell malignancies[14,15].

In leukemic cell lines, bortezomib was shown to interact in an additive or synergistic way when combined with traditional drugs, including glucocorticoids[16]. In pre-clinical T-ALL mouse models, bortezomib showed modest single-agent activity[17], while almost no

monotherapy activity was observed in phase I studies in children and adults[8,9]. However, phase I or phase II studies in which bortezomib was combined with conventional chemotherapeutics showed promising clinical activity in adult AML patients[18] and pediatric ALL patients[10,19].

Despite the encouraging results of bortezomib in several hematological malignancies, emergence of bortezomib resistance as well as side effects are factors that limit its long-term efficacy[20–22]. To overcome these issues, several irreversible proteasome inhibitors have been developed[23]. Carfilzomib is more selective for the proteasomal chymotrypsin-like activity and is a more effective anti-leukemic drug at low concentrations than bortezomib[24]. Subsequently, an orally bioavailable analogue of carfilzomib was developed, ONX 0912, which elicited anti-tumor activity by inhibiting chymotrypsin-like activity in Waldenstrom macroglobulinemia[15], and MM[25]. Upon recognition of the important role of immunoproteasomes, ONX 0914 was developed as the first β5i selective proteasome inhibitor[1,3]. Alternatively, to overcome bortezomib-resistance, proteasome inhibitors that target other non-catalytic parts of the proteasome may be attractive. In this context, the non-competitive proteasome inhibitor 5-amino-8-hydroxyquinoline (5AHQ) may serve as a prototypical drug that binds the structural α7 subunit of the proteasome and induces cell death of *in vitro* established bortezomib-resistant hematological cell lines[26].

The aim of the current study was to examine the *ex vivo* sensitivity of pediatric leukemia cells (ALL and AML) to 1) bortezomib as a single agent and in combination with dexamethasone, and to 2) next generation epoxyketone-based irreversible proteasome inhibitors designed to overcome bortezomib resistance. To identify novel parameters that may predict proteasome inhibitor response, we explored whether or not their cytotoxic activity correlated with protein expression levels of the constitutive subunits β5, β1, β2, and α7, and the immunoproteasome subunits β5i, β1i and β2i. We show that higher ratios of immune *vs.* constitutive proteasome level represent a novel indicator of sensitivity of pediatric acute leukemia cells to bortezomib and epoxyketone-based proteasome inhibitors.

METHODS

Leukemic patient samples

Forty-four pediatric leukemia samples (12 AML and 32 ALL samples) were included in this study. Table 1 depicts an overview of patient characteristics. After thawing the vials, viable cells were counted and blast percentage was determined after May-Grunwald/Giemsa cytospin stainings. Inclusion criteria for the MTT assay were that more than 80% blasts were present in the leukemic samples. These non-proliferating cells were immediately used for MTT analysis, and the remaining cells were snap-frozen for proteasome subunit protein expression.

Table 1. Patient characteristics

		ALL	AML
Age, years mean ± SD		5.6 ± 4.3	8.3 ± 5.5
Female		18 (44%)	7 (42%)
Male		14 (56%)	5 (58%)
WBC median, 10^9/L		5.1 (range 2-1332)	25.5 (range 5-307)
Subtype	Common	13	
	Pro-B	4	
	Pre-B	9	
	T-ALL	5	
	Unknown	1	
FAB type	M1		2
	M2		3
	M3		1
	M4		1
	M5		3
	M6		1
	Unknown		1

WBC: white blood cell count

MTT cytotoxicity assay

Cytotoxicity of bortezomib, dexamethasone, as well as their combination, and carfilzomib, ONX 0912, ONX 0914, and 5AHQ was determined using the MTT colorimetric dye reduction assay[27]. For the drug combination study, CalcuSyn (Version 1.1.1 1996, Biosoft, Cambridge UK) software was used to calculate a combination index (CI) based on the median-effect principle, for each drug combination tested[28]. For detailed information see the supplemental document.

Protein expression

Western blotting

Protein expression was determined by Western blot analysis, as previously described[20]. Protein bands were quantified by Odyssey software, corrected for background, and normalized with β-actin to correct for any loading differences. To compare between gels, subunit expression of the patient samples were normalized using subunit expression in the leukemic T-ALL cell line CCRF-CEM[20]. Since each antibody has different binding characteristics, it is not possible to determine ratios between the expression of each different subunit using Western blot analysis.

ProCISE analysis

A previously described ELISA-based method (ProCISE) was used to confirm the Western blot data and to accurately quantify the fraction of constitutive and immunoproteasome subunits per patient[29]. Details on validation and statistical evaluation are described in the supplemental document.

Statistical analysis

Statistical significance of the differences between subunit expression and differences in drug sensitivity for AML and ALL patients was determined using the Mann-Whitney U test. Correlations of subunit expression with drug LC_{50} concentrations were calculated by Spearman correlation. Statistical significance was achieved when $P<0.05$. All statistical analyses were performed using SPSS (version 20.0).

This study has been approved by the Local Ethics Committee VUmc. Date of approval: December 5, 2000. Approval file number: TJFS/bz2568a

RESULTS

Constitutive proteasome and immunoproteasome composition in childhood ALL and AML cells

Quantitative assessment of total proteasome, constitutive- and immunoproteasome by ProCISE analysis was performed on 19 childhood ALL and 6 childhood AML samples (Figure 1). We refer to immunoproteasomes as forms composed of β5i, β1i and β2i subunits, although ProCISE assay could also identify β5i and β1i subunits being incorporated in hybrid proteasome forms[12]. Total proteasome and constitutive proteasome subunit levels did not differ significantly between ALL and AML, however, immunoproteasome expression was significantly higher in ALL over AML (19 *vs.* 12.8 ng/μg total protein, p=0.036). Notably, total immunoproteasome levels outweigh constitutive proteasome levels by at least 2-3 fold (Figure 1A), which is also manifested in expression levels of individual immunoproteasomes and constitutive proteasomes (supplemental Figure S1; Table S1).

Next, we determined the individual ratios of immunosubunit over constitutive subunit expression. For ALL and AML the highest ratio was observed for β5i/β5 and lowest ratio for β2i/β2. Ratios of β2i/β2 were significantly (p=0.043) increased in ALL vs. AML samples whereas a trend for higher β1i/β1 was noted (p=0.059) (Figure 1B). Furthermore, we examined the impact of variations in constitutive- and/or immunoproteasome expression for total cellular proteasome expression levels (Figure 1C). In contrast to constitutive subunit expression, increasing immunoproteasome subunit expression was significantly correlated with increased total proteasome levels in both ALL and AML cells.

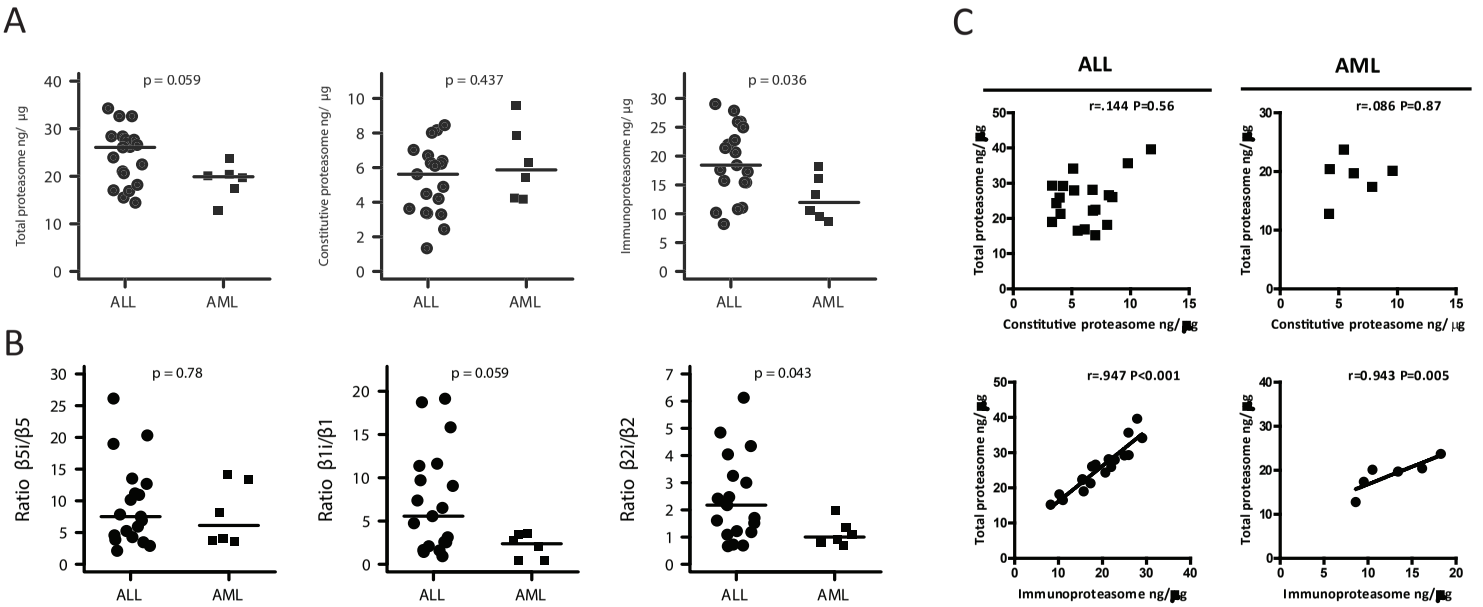


Figure 1. Proteasome expression of ALL and AML patient samples determined by ProCISE assay. **(A)** Total proteasome levels and subdivision of constitutive- and immunoproteasome levels (ng/µg total protein) in childhood ALL and AML cells. The total value of constitutive proteasome and immunoproteasome expression for each patient is shown and depicts the variation between patients. **(B)** The ratio of paired subunits (between β5i and β5 and between β1i and β1) is shown within each patient sample. The line denotes the mean for ALL (n=19) and AML (n=6). **(C)** Correlation plots of total proteasome as a function of constitutive proteasome, and total proteasome as a function of immunoproteasome levels. The individual subunit data are shown in Supplemental Figure 1.

The above described analyses were further extended by comparing subgroups of ALL (BCP-ALL and T-ALL) for constitutive- and immunoproteasome expression and ratios of individual immunoproteasome over constitutive proteasome (supplemental Figure S2). Although the sample size was limited, BCP-ALL, T-ALL and AML displayed similar levels of constitutive proteasome, but total immunoproteasome levels were higher in BCP-ALL (4-fold), AML (2-fold) and T-ALL (2-fold) compared to constitutive proteasome levels (supplemental Figure S2A). Further dissection of the ratios of β5i/β5, β1i/β1 and β2i/β2 expression in ALL subgroups revealed high ratio of β5i/β5 the BCP-ALL subgroup (ratio: 7.1) and AML (ratio: 7.9) compared to T-ALL (3-fold) (supplemental Figure S2B). β1i/β1 ratios were high for BCP-ALL (ratio: 7.4) compared to T-ALL and AML (2-fold) and β2i/β2 ratios were consistently the lowest among all leukemia subgroups. Further dissection of BCP-ALL in pro-B-ALL, pre-B-ALL and common-ALL revealed high ratios of β5i/β5 in all subgroups, with β1i/β1 ratios being particularly low in pro-B-ALL, T-ALL, and AML (supplemental Figure S2C). Due to small subgroup-size, no statistics were applied on these subgroup comparisons. For comparison, supplemental Figure S2C shows values of ratios for established *in vitro* cell line models of human T-ALL (CCRF-CEM) and AML (THP1).

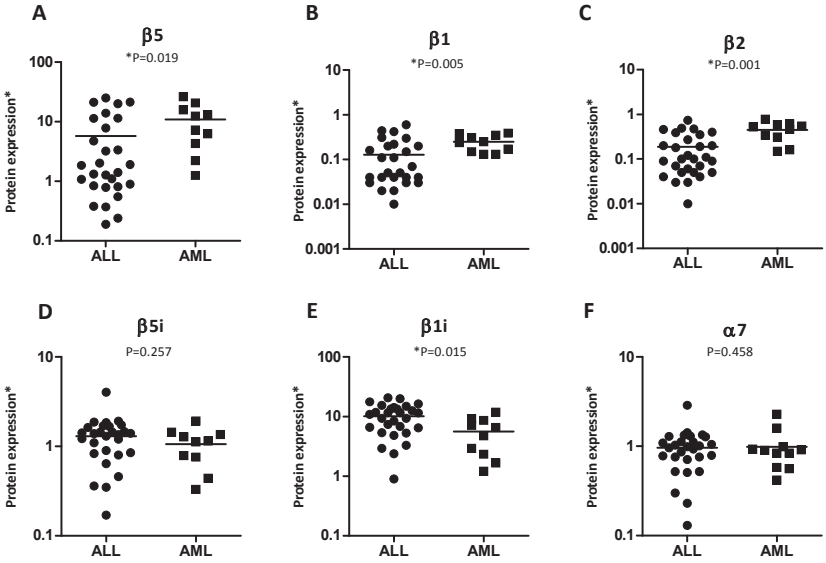


Figure 2. Proteasome protein subunit expression in ALL versus AML determined by Western blotting. Comparison of proteasome subunit expression of constitutive subunits; **(A)** β5, **(B)** β1, **(C)** β2, and immunoproteasome subunits; **(D)** β5i and **(E)** β1i, and **(F)** structural subunit α7 in ALL and AML patient samples. Protein expression determined by Western blotting was normalized on β-actin as loading control and to subunit expression of CCRF-CEM cell line as control between blots. Note that these data depict relative quantifications of subunit expression, whereas ProCISE analysis provides absolute quantification of subunits. The lines represent the mean.

The preliminary account shown in supplemental Figure S1 suggested that differences in immuno/constitutive proteasome ratios between ALL and AML (Figure 1B) were associated with increased constitutive proteasome levels and decreased immunoproteasome levels in AML *vs*. ALL cells. These observations were confirmed in a large group of ALL and AML patient samples (n=29 and n=12, respectively) by Western blot analysis of relative levels of immunoproteasomes and constitutive proteasomes, normalized on housekeeping gene β-actin and cell line CEM. Please note that these data depict relative quantifications of subunit expression, whereas ProCISE analysis provides absolute quantification of subunits. Figure 2 shows significantly increased levels of constitutive β5, β1 and β2 subunit levels in AML *vs*. ALL samples, whereas AML cells had significantly lower levels of β1i and a tendency towards lowered β5i levels compared to ALL cells. No significant differences in expression level of the non-catalytic α7 subunit were observed. Upon classification of ALL samples into subgroups, pro-B ALL (n=4) and T-ALL (n=4) samples expressed relatively higher β5, β1, and β2 constitutive subunit expression levels than both pre-B ALL (n=7) and common-ALL (n=10), whereas there was a trend for the reverse with respect to β5i and β1i expression (supplemental Figure S3). Taken

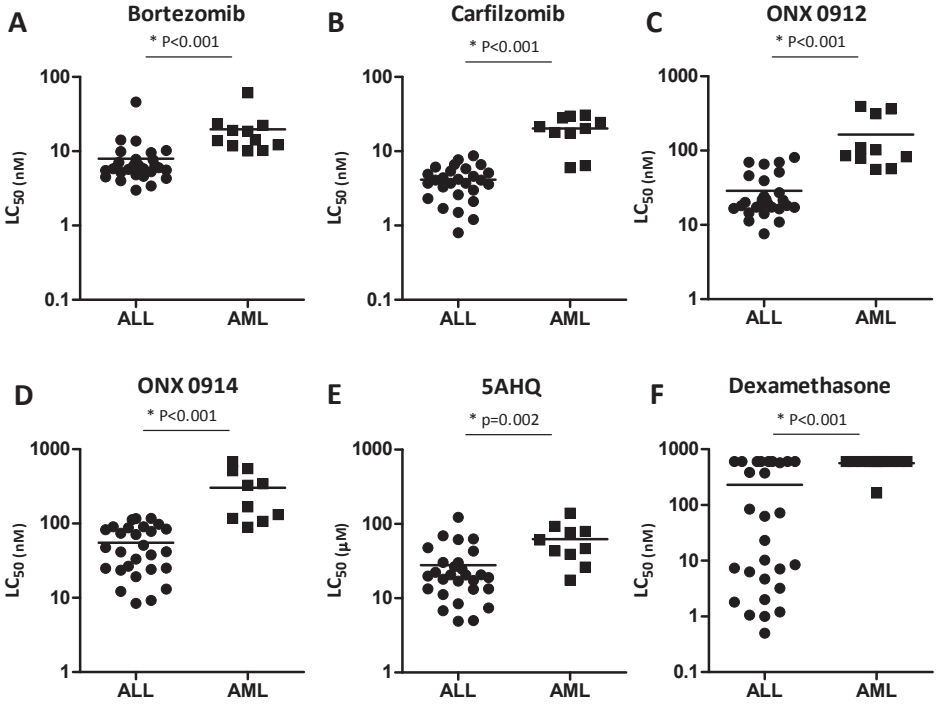


Figure 3. Sensitivity of pediatric ALL and AML patient samples to proteasome inhibitors and dexamethasone. Comparison of LC_{50} values obtained by MTT cytotoxicity assay after 96 hours exposure to; **(A)** bortezomib, **(B)** carfilzomib, **(C)** ONX 0912, **(D)** ONX0914, **(E)** 5AHQ, and **(F)** dexamethasone in ALL and AML patient samples. The linesrepresent the mean and the y-axis is depicted as a logarithmic scale.

altogether, the results demonstrate that proteasome composition in pediatric ALL and AML cells is largely represented (>70%) by immunoproteasome subunits and to a minor extent (<30%) by constitutive subunits. Beyond this, ratios of individual immuno/constitutive proteasomes varies considerably mostly in the order of β5i/β5 > β1i/βi > β2i/β2, of which the impact on response to constitutive- and immunoproteasome inhibitors warrants further exploration.

Growth inhibitory effect of proteasome inhibitors against primary pediatric ALL and AML cells

Ex vivo sensitivity of pediatric leukemia patient cells towards different proteasome inhibitors was assessed in 4-day cytotoxicity assays (Figure 3). Apart from sensitivity to bortezomib, drug sensitivity was also determined for 3 epoxyketone-based irreversible proteasome inhibitors (carfilzomib, ONX 0912 and ONX 0914) and 5AHQ. With a median LC_{50} of 14.0 nM (range: 10.1-61.0 nM), AML samples were significantly (P<0.001) less sensitive to bortezomib than ALL cells (median $LC_{50:}$ 6.0 nM, range: 3.0-46.1 nM) (Figure 3A). Figure 3B,E and supplemental Table S1 summarize the sensitivity to the individual proteasome inhibitors as median LC_{50} values. Statistical comparisons between proteasome inhibitor sensitivity of AML and ALL samples consistently revealed that ALL cells were the most drug sensitive (p<0.001). Notably, ALL samples were markedly sensitive to carfilzomib (Figure 3B) with a median LC_{50} of 4.1 nM, hence carfilzomib being 1.5-fold more potent than bortezomib (LC_{50}: 6 nM). Again, AML samples were significantly more resistant to carfilzomib (median LC_{50}: 20.8 nM) compared to ALL. Sensitivity for ONX 0912, an oral analogue of carfilzomib, remained in the low nanomolar range for ALL (median LC_{50}: 19.2 nM) and 4-fold lower than for AML cells (median LC_{50}: 93.7 nM) (Figure 3C). The largest difference (5.6-fold) between ALL and AML cells was observed for the immunoproteasome inhibitor ONX 0914 (median LC_{50}: 44.6 nM vs. 248.0 nM) (Figure 3D). Finally, ALL samples displayed 2.5-fold greater sensitivity for 5AHQ (median LC_{50}: 20.1 μM vs. 53.8 μM) compared to the AML samples (Figure 3E). As reported previously[30], the latter cells were also the least sensitive to dexamethasone (Figure 3F). Within the ALL subgroups, pre-B-ALL and common-ALL samples appeared to be most sensitive for carfilzomib, ONX 0912, ONX 0914 and 5AHQ as compared to pro-B-cells (supplemental Figure S4).

Combination effects of bortezomib and dexamethasone

To investigate possible synergistic effects of combination therapy, dexamethasone was combined with bortezomib. Towards this end, pediatric leukemia samples were first tested for dexamethasone as single agent in MTT assays (Figure 3F). As expected and consistent with previous studies[31], ALL samples were markedly more sensitive to dexamethasone than AML samples (median LC_{50}: 62.4 nM vs. >600 nM, P<0.001). Within

the group of AML samples, 10 out of 11 (91%) had an LC_{50}>6 μM, which is the case in only 31% of ALL samples. Based on the dose-response curves of bortezomib and dexamethasone alone, 4 different concentrations of bortezomib were selected (range 2.4-20 nM) in combination with 5 concentrations of dexamethasone (range 0.18-750 nM). Nineteen ALL and 7 AML samples could be used for further calculation of a CI based on the median-drug effect analysis[28]. Notably, the highest synergy was found for combinations with dexamethasone in the low bortezomib concentration range (2.4-11.8 nM) (Figure 4A). Furthermore, all 5 dexamethasone-resistant (defined as less than 50% cell kill) ALL patients displayed sensitivity for bortezomib as well as synergism in combination with dexamethasone. The only ALL patient with *ex vivo* bortezomib-resistance was sensitive for dexamethasone and to the bortezomib-dexamethasone combinations. Remarkably, bortezomib sensitized all 6 dexamethasone-resistant AML patients for dexamethasone. Lastly, the combination of dexamethasone and bortezomib was synergistic in the samples of 4 AML patients being resistant to both dexamethasone and to bortezomib. Regardless of dexamethasone concentration used, 11.8 nM bortezomib established the most synergistic combinations and the highest fraction affected for AML. Overall, bortezomib was most sensitizing in dexamethasone-resistant cells, which may be of potential clinical relevance since these patients have a dismal prognosis.

As a comparison, the T-ALL cell line CCRF-CEM was exposed to combinations of bortezomib and dexamethasone for 4 days, either with or without prior pulse exposure to 1 μM bortezomib for 1 hour to mimic *in vivo* peak plasma pharmacokinetic concentrations of bortezomib followed by steady state plasma levels of 10-20 nM bortezomib. CEM cells

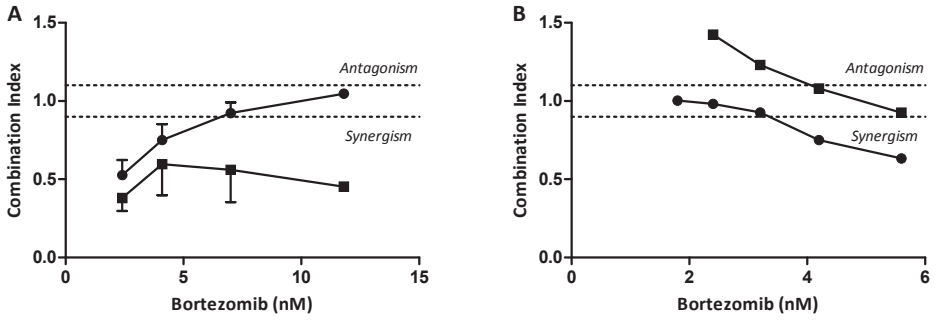


Figure 4. Combination Indices of bortezomib-dexamethasone combinations. (A) Mean CI of 3 representative dexamethasone concentrations per bortezomib concentration in patient samples. Symbols represent ALL (●) and AML (■). For ALL (n=17), 1.5 nM, 11.7 nM, and 93.8 nM dexamethasone were selected, and for AML (n=6), 11.7 nM, 93.8 nM, and 750 nM dexamethasone were used. Antagonism (CI > 1.1) additivity (CI > 0.9 < 1.1), and synergism (CI < 0.9). Error bars represent standard error of the mean of 3 separate experiments. **(B)** Combination indices of T-ALL cell line CEM exposed to combinations of bortezomib and dexamethasone. Symbols represent (●) CEM pulsed for 1 hour with 1 μM bortezomib prior to the combination assay and (■) CEM control, not pulsed prior to the combination assay. Experiments were performed twice in triplicate. Results of the mean of these experiments are presented.

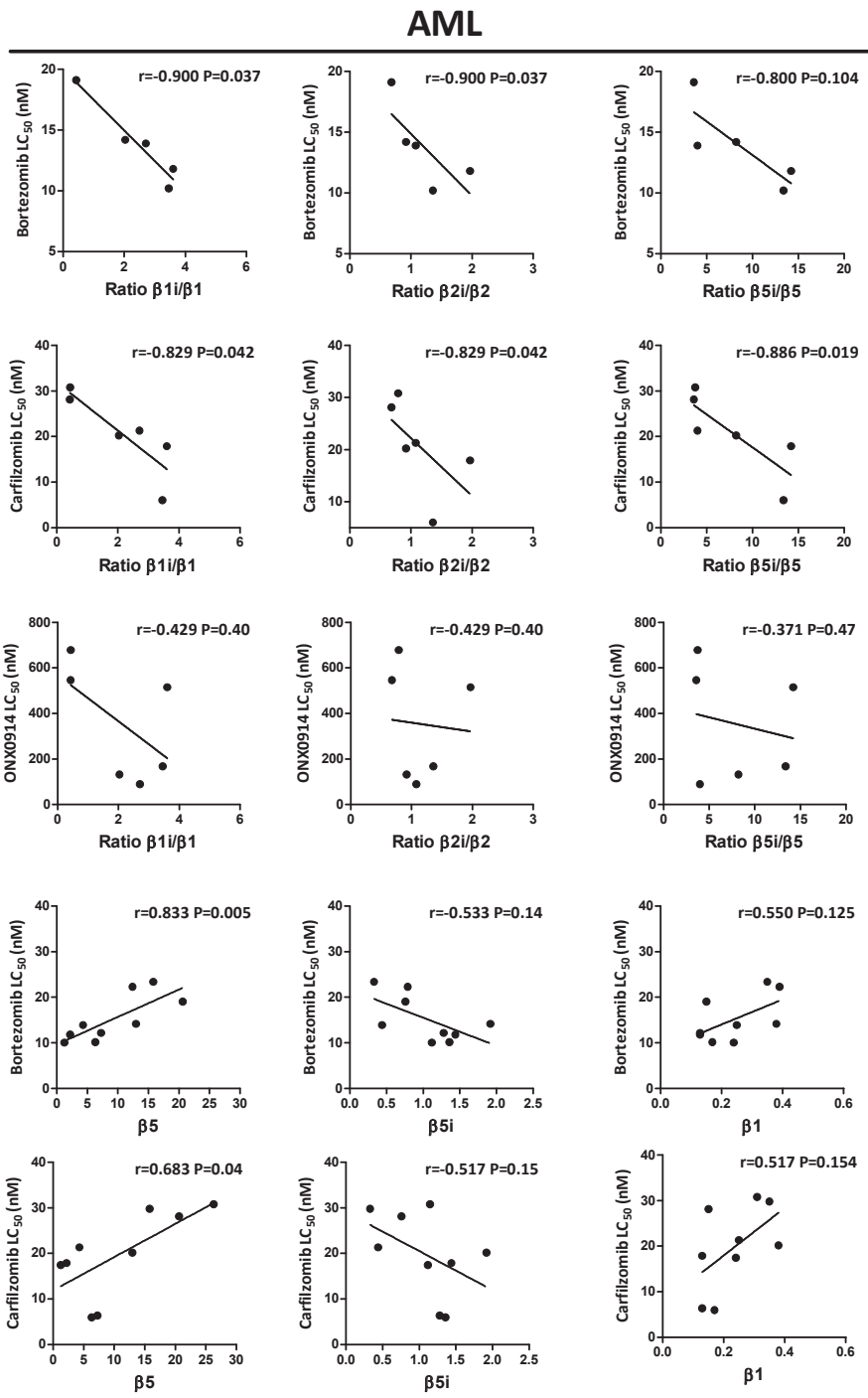
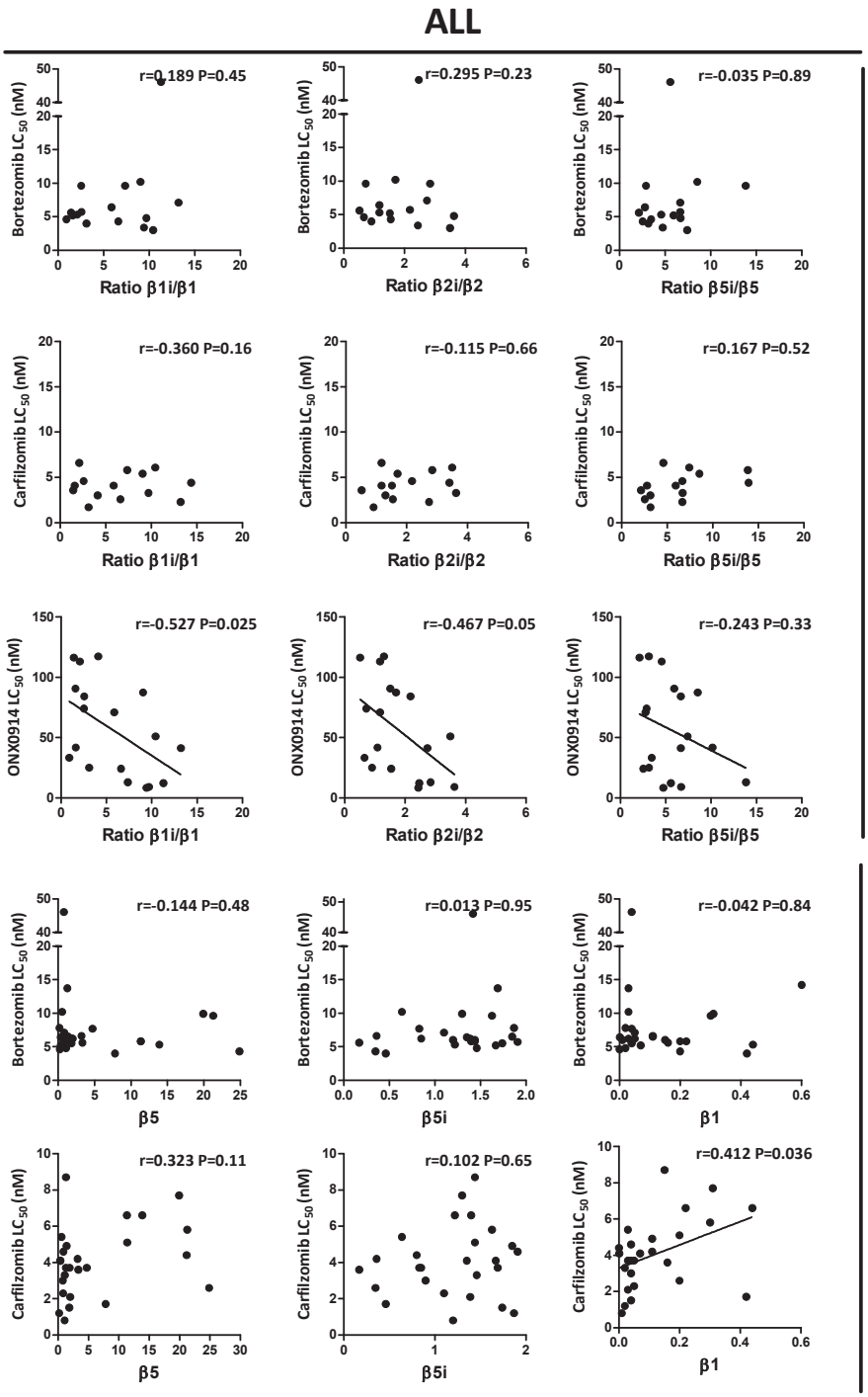


Figure 5. Representative correlations of proteasome inhibitor sensitivity and constitutive and immunoproteasome subunit expression in pediatric ALL and AML.



Correlations of ratios (β1i/β1, β2i/β2 and β5i/ β5) determined by ProCISE with LC_{50} values (bortezomib, carfilzomib, ONX 0914) for AML and ALL patient samples. Correlations of single subunits with LC_{50} values of bortezomib and carfilzomib for AML and ALL patient samples with β5, β5i, and β1.

that were pulse-treated with bortezomib showed increased sensitivity to dexamethasone (IC_{50}: 18 nM) compared to CEM cells that were not pulse-treated with bortezomib (IC_{50}: 24 nM). Furthermore, combination experiments in the clinically achievable concentration range of bortezomib and dexamethasone showed three additive (CI: 0.97±0.04) and two synergistic combination indices (CI: 0.69±0.08) in the pulse-treated T-ALL CEM cells (Figure 4B).

Correlates of proteasome subunit expression and sensitivity to proteasome inhibition

Recently, *in vitro* studies indicated that upregulation of the constitutive subunits was associated with decreased bortezomib sensitivity[20,22,32,33]. Here we investigated whether differential expression levels of constitutive and/or immunoproteasome levels may underlie responsiveness to bortezomib and other proteasome inhibitors. Interestingly, ratios of immune/constitutive proteasome revealed most significant correlations or consistent trends such that increased ratios correlated with increased sensitivity to bortezomib and carfilzomib in AML, and ONX 0914 in ALL patients. This is illustrated in Figure 5, where sensitivity for bortezomib in AML inversely correlated with the ratios β1i/β1 and β2i/β2 (r=-0.900, p=0.037) whereas a trend (r=-0.800, p=0.104) was noted for β5i/β5. In addition, all three ratios correlated significantly with sensitivity for carfilzomib. For ALL, no significant correlations were revealed with bortezomib- or carfilzomib-sensitivity, however, β1i/β1 ratio correlated significantly with sensitivity for ONX 0914 (r=-0.527, p=0.025) and a trend for correlation with β2i/β2 was seen (r=-0.467, p=0.05). Overall, supplemental Table S2 summarizes that for AML samples all proteasome inhibitors, except 5AHQ, displayed lower LC_{50}'s when immuno/constitutive proteasome ratio was higher. In ALL samples this was true for 11 out of 15 proteasome inhibitors and immuno/constitutive proteasome ratio combinations. Other selected examples of correlates that were observed based on Western blot analysis of constitutive- and immunoproteasome levels in AML and ALL samples are shown in Figure 5. Carfilzomib and bortezomib LC_{50} values for AML correlated positively with β5 and β1 expression and inversely with β5i. For the same drugs in ALL samples, no such correlations were found.

Collectively, these results indicate that immuno/constitutive proteasome ratios, but not the mere expression of constitutive levels *per se*, is a novel correlative factor in response to proteasome inhibitors in pediatric ALL and AML cells, with increased ratios being predictive of increased sensitivity to proteasome inhibitors.

Effect of PSMB8 silencing on proteasome inhibitor sensitivity in THP1 cells

To study the role of the immunoproteasome subunits in the sensitivity to proteasome inhibitors at a mechanistic level, THP1 cells were pre-exposed to *PSMB8* siRNA or *PSMB9* siRNA. After 24h of siRNA (mean knockdown of 3 experiments: 76%, Figure S5A), 4-day

MTT assays were performed with bortezomib and ONX 0914 to assess cell growth inhibitory effects. *PSMB8* siRNA had no significant impact on the intrinsically high bortezomib-sensitive phenotype of THP1 cells (IC_{50} 3.2±0.5) compared to non-target siRNA (IC_{50} 3.6±0.3). For ONX 0914, however, siRNA *PSMB8*-silencing was accompanied with a 2.1-fold loss of sensitivity (IC_{50}: 71.4±5 nM) compared to non-target siRNA controls (IC_{50}: 34.2±7 nM). Silencing *PSMB9* had no effect on the sensitivity to both proteasome inhibitors (Figure S5B).

DISCUSSION

Proteasome inhibitors, particularly bortezomib, are being explored in the clinical setting in childhood acute leukemias[10]. Unfortunately however, chemoresistance to bortezomib may occur that could hinder its pharmacologic activity. In this respect, several *in vitro* studies with human leukemia cell lines identified mechanisms of acquired bortezomib resistance, most frequently due to upregulation of the proteasomal β5 subunit[20,22,32–34] as well as acquisition of β5 subunit mutations that decrease bortezomib binding[20,22,34,35]. The current research is the first to address these proteasome-based drug sensitivity and resistance phenomena in a relatively large series of childhood acute leukemia patient specimens and in particular studying the immunoproteasome as a novel druggable target. Although the patient samples evaluated in the current study displayed differential bortezomib sensitivity, this observation was not associated with mutations in the β5 subunit of the proteasome[20] (results not shown). This may have been anticipated as all of the samples were initial diagnosis leukemia samples, and β5 mutations found in human leukemia cell lines were typically acquired after prolonged bortezomib exposure. In a clinical setting, though studies are limited, no *PSMB5*-associated mutations were found in patients treated with bortezomib[36,37]. Rather, these and other studies[20,33] point to upregulation of β5 subunits expression as a primary response mechanism to bortezomib, which may set a stage for acquisition of mutations following prolonged bortezomib exposure. The present study established that ALL cells were significantly more sensitive to bortezomib as a single agent than AML cells. Interestingly, even the prognostically unfavorable subgroups pro-B ALL and T-ALL, which are resistant to most chemotherapeutic drugs[38], were equally sensitive to bortezomib as pre-B/common ALL samples. This is underscored in a study by Szczepanek et al[39], who showed that bortezomib was even more potent in T-ALL patient samples compared to pre-B/common ALL. In this study, bortezomib was found to be a potent drug for this therapy-resistant subgroup of ALL although the 2 T-ALL patients treated in a phase II clinical trial with bortezomib did not reach complete remission[10]. Clearly, larger clinical studies with bortezomib in this hematological malignancy are warranted.

Consistent with sensitivity patterns for other anti-leukemic drugs, ALL cells were also more sensitive to dexamethasone than AML cells. Sensitivity to glucocorticoids is an important prognostic marker for therapy response of ALL patients[7]; hence the synergistic combination of dexamethasone and bortezomib may improve therapy response and decrease disease recurrence rates. Although the molecular basis for glucocorticoid resistance in AML patients remains elusive[40], our data showed that bortezomib can sensitize cells for dexamethasone-induced growth inhibition. In particular, the specimens of 4 AML patients resistant to bortezomib and dexamethasone were sensitive to the combination of these drugs (mean CI: 0.51). For ALL, bortezomib and dexamethasone combinations displayed a lesser synergistic effect probably due to the fact that ALL samples are intrinsically more sensitive to either bortezomib or dexamethasone alone. Consistent with this notion, dexamethasone resistant ALL samples could indeed be further sensitized by bortezomib/dexamethasone combinations as previously described by others[41] for (pre)clinical studies with MM cells. In fact, low dose dexamethasone and bortezomib combinations were synergistic in 88% of ALL patients, and in all 5 AML patients. From a mechanistic perspective, the sensitization of dexamethasone-resistant AML cells by bortezomib might be possibly achieved via suppression of constitutively active NF-κB in AML cells[42], thereby triggering dexamethasone-mediated apoptosis. Consistently, combination experiments in T-ALL CEM cells also showed additive/synergistic effects at low nanomolar combinations of bortezomib and dexamethasone, even with short pre-exposures to high bortezomib concentrations mimicking peak plasma pharmacokinetic concentrations.

Given the emergence of bortezomib-resistance phenomena and untoward toxicity after chronic bortezomib administration, next generation irreversible proteasome inhibitors were developed to overcome these problems[4,23]. In this respect, we tested a series of novel proteasome inhibitors in comparison with bortezomib to determine their efficacy in leukemic cell kill. ALL cells were more sensitive to these epoxyketone-based proteasome inhibitors than AML cells. For ALL patient samples, carfilzomib was most potent, followed by bortezomib, ONX 0912, ONX 0914 and 5AHQ. Of note, this drug-sensitivity pattern of ALL patient samples actually follows that of the human T-ALL cell line CCRF-CEM[20].

The differential sensitivity of ALL *vs.* AML cells for both bortezomib and epoxyketone-based proteasome inhibitors may, in part, be due to common mechanisms mediating drug resistance. Constitutively active NF-κB in AML cells[42] can attenuate the induction of apoptosis, but also upregulate the expression of the dominant multidrug efflux transporter MDR1/P-glycoprotein (Pgp/ABCB1)[43]. High Pgp expression has been associated with poor outcome in acute leukemia[44]. Although bortezomib is considered to be a poor substrate for Pgp[22,45], the expoxyketone-based proteasome inhibitors carfilzomib, ONX 0912 and ONX 0914 were found to be *bona fide* substrates of Pgp[45]. This notion, together

with the fact that MDR1/Pgp expression is increased in AML cells over ALL cells[46], may result in a reduced sensitivity of AML cells to epoxyketone-based proteasome inhibitors.

Both ALL and AML patient cells displayed the lowest sensitivity to the immunoproteasome inhibitor ONX 0914, although effective drug concentrations still fall within the nanomolar range. This may be counterintuitive as pediatric acute leukemia cells do express appreciable levels of immunoproteasomes including β5i, the main target of ONX 0914. Hence, this result appears to be consistent with the outcome of a study by Parlati et al[14] indicating that beyond the inhibition of the β5i subunit, inhibition of additional subunits including β5 and β1, is required to elicit a superior anti-leukemic response.

Several attempts have been made in tumor cell line models to correlate differential bortezomib sensitivity to proteasome expression levels. Busse et al[47] showed that bortezomib-resistant solid tumor cell lines expressed lower levels of immunoproteasome than the more sensitive hematologic B-cell lines. Moreover, all cell lines classified as most bortezomib-resistant showed low β1i and/or β2i mRNA expression compared to bortezomib-sensitive cell lines. Hematological cell lines with acquired resistance to bortezomib were also characterized with increased levels of constitutive proteasome levels and reduced levels of immunoproteasome[20,22]. In two clinical studies, Matondo et al[48] suggested a relationship between 20S proteasome levels and sensitivity to proteasome inhibitors in AML patient samples, although no distinction between constitutive- and immunoproteasome subunit expression was made. Shuqing et al[37] described mRNA overexpression of β5 in a single MM patient (without a mutation in *PSMB5)* with clinical resistance to bortezomib-containing therapy compared to 3 sensitive patients. Together, these findings would imply that leukemic patients harboring higher constitutive (and lower immunoproteasome) subunit expression before bortezomib treatment would display a poorer response to bortezomib-based treatment than patients who have lower constitutive proteasome subunit expression. In the present study, ProCISE and Western blot analysis allowed the correlation of *ex vivo* drug-sensitivity to constitutive- and immunoproteasome subunit expression in ALL and AML cells.

Although total proteasome levels did not differ significantly between ALL and AML samples, we found that ALL cells had significantly lower expression of constitutive proteasome subunits and higher β1i expression than AML cells. Consistent with the above concept, increased bortezomib- and carfilzomib-sensitivity of AML cells correlated with lower β5 expression and with higher β5i expression. These correlations were further corroborated for ONX 0914 by demonstrating that sensitivity to this drug was observed with increasing ratios of immuno/constitutive proteasome in ALL. Since the ALL samples were all relatively sensitive for bortezomib and carfilzomib, no significant correlations were revealed with these drugs. The *ex* vivo part of this study has however, some limitations such as relatively low numbers of patients per subgroup while performing many correlation analyses and the lack of mechanistic work due to limited number of cells

available. Notwithstanding these facts, several mechanistic data support the notion that modulation of expression levels of individual β5i and β5 subunits has an impact on (immuno) proteasome inhibitor sensitivity, e.g. β5i knockdown in THP1 cells abrogates sensitivity to ONX 0914 (Suppl. Fig S5) whereas interferon-γ induced upregulation of β5i sensitized for ONX 0914[49], and β5 knockdown in THP1 cells increased bortezomib sensitivity[22].

To establish whether the immuno/constitutive proteasome ratio represents an additional contributing factor in proteasome inhibitor response would deserve further investigations in larger size patient cohorts. It will be critical to further decipher the mechanisms regulating proteasome homeostasis and in particular the equilibrium between the assembly of the immunoproteasome *vs.* constitutive proteasome in AML and ALL (subgroups). Furthermore, it is important to mention that in this study we refer to the classical definition of immunoproteasomes as those composed of β5i+β1i+β2i subunits, but acknowledge that an unknown fraction of β1i and β5i subunits can be assembled in hybrid proteasome forms[12]. It will be of interest to explore how these proteasomes variants contribute to inhibition profiles by proteasome inhibitors. In this context, it is interesting to note that immunoproteasomes rather than constitutive proteasome levels were dictating total proteasome levels in AML and ALL cells, which may provide a mechanistic rationale[50] for the targeting of immunoproteasomes in order to disrupt proteasome homeostasis and elicit an anti-leukemic response.

In conclusion, bortezomib displayed potent cytotoxic effects against pediatric ALL and AML cells. This pharmacological efficacy was further enhanced in combination with dexamethasone, eliciting additive or synergistic effects. ALL cells were intrinsically more sensitive to proteasome inhibitors than AML cells, for which higher ratios of immuno/constitutive proteasome was an accountable factor. Thus, for next generation proteasome inhibitors including immunoproteasome inhibitors, these findings may hold promise in the future treatment of pediatric leukemia by avoiding toxicity of bortezomib, circumvention of bortezomib resistance and further assessment of their synergistic effect when combined with other drugs including glucocorticoids.

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Authorship contribution and conflict of interest statements

Contribution: D.N., N.E.F., J.M., and J.A. performed experiments; C.J.K., A.D.S., and V.H. provided materials or patient samples; D.N., J.D., G.J., and J.C. analyzed results, performed

statistical analyses and prepared the figures; D.N., N.E.F., Y.G.A., S.Z., G.J., G.J.L.K., and J.C. designed the research and wrote the paper. N.E.F., Y.G.A., C.J.K., A.D.S., S.Z., V.H., T.H., and G.J.L.K. discussed the format and content of the article and contributed to the review and editing of the final manuscript.

Conflict-of-interest disclosure: J.D. and J.A. are employees of Onyx Pharmaceuticals. C.J.K. is employee and shareholder of Onyx Pharmaceuticals. All other authors declare no competing financial interests.

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SUPPLEMENTAL MATERIALS AND METHODS

Antibodies and Reagents

Dexamethasone sodium phosphate was obtained from the VU University Medical Center pharmacy. BTZ was kindly provided by Millennium Pharmaceuticals (Cambridge, MA, USA). 5-Amino-8-Hydroxyquinole (5AHQ) was synthesized by Dr A.D. Schimmer (Toronto, Canada)[26]. The epoxyketone-based PIs CFZ, 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 ß1, ß2, ß5, ß1i, ß5i, 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% CO2 and 37 °C. Cell cultures were seeded at a density of 3x105 cells/ml and refreshed twice weekly.

MTT cytotoxicity assay

For drug combination studies, ALL ($2x10^6$ cells/ml), or AML cells ($1x10^6$ cells/ml) were incubated with 5 different fixed concentrations of single drug BTZ or DEX and their combination in 150 µl medium in a 96-wells plate for 96 hours at 370C. To determine which concentrations of DEX and BTZ were optimal for drug combination assays, 8 patient samples were initially tested for a wide range of concentrations. For single-drug DEX, an 8-fold dilution range of 6 µM - 0.18 nM was tested. For single-drug BTZ, 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 BTZ were combined with a 5-fold-dilution range of DEX (750 nM - 0.18 nM). Cells were pre-incubated with freshly prepared BTZ for 2 hours at 37^0C and then added to a 96-wells plate containing different DEX 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.

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. The LC_{50} concentrations were determined in patient specimens after 4 days drug exposure. Mutually non-exclusive CIs were used for experimental values, because DEX and BTZ 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 × 106 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 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 MgCl2, 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(Ct target-Ct GUS) × 100%.

SUPPLEMENTAL TABLES

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

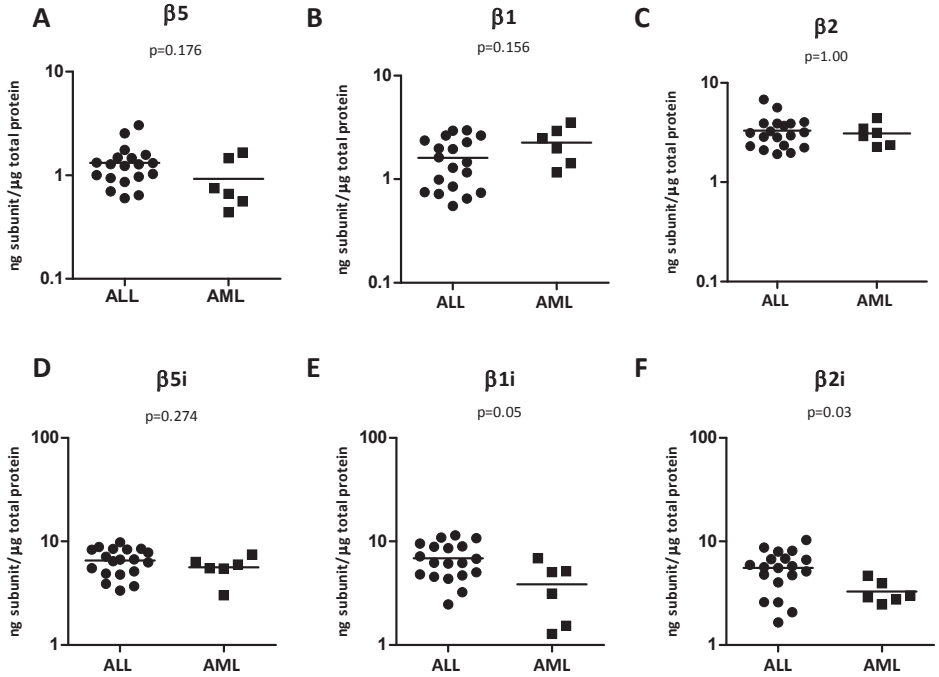
	ALL			AML			
Pro-CISE	**N**	**Median expression ng/µg total protein**	**Range**	**N**	**Median expression ng/µg total protein**	**Range**	***P***
ß5	19	1.27	0.6 - 3.04	6	0.71	0.44 - 1.66	0.176
ß5i	19	6.66	3.34 - 9.81	6	6.16	3.02 - 7.46	0.274
ß1	19	1.46	0.55 - 2.69	6	2.24	1.16 - 3.53	0.156
ß1i	19	6.21	2.46 - 11.4	6	4.10	1.28 - 6.87	0.05
ß2	19	3.13	1.91 - 6.77	6	3.03	2.26 - 4.41	1.00
ß2i	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*
ß5	28	1.63	0.19 - 24.9	10	9.8	1.25 - 26.3	0.006
ß5i	28	1.37	0.17 - 4.03	10	1.1	0.33 - 1.92	0.453
ß1	29	0.05	0.00 - 0.60	10	0.24	0.13 - 0.39	0.000
ß1i	29	10.9	0.90 - 20.7	10	5.7	1.21 - 11.9	0.037
ß2	29	0.1	0.01 - 0.74	10	0.49	0.15 - 0.78	0.000
ß2i	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*
BTZ	30	6.0	3.0 – 46.1	11	14.0	10.1 – 23.4	0.000
CFZ	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
DEX	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

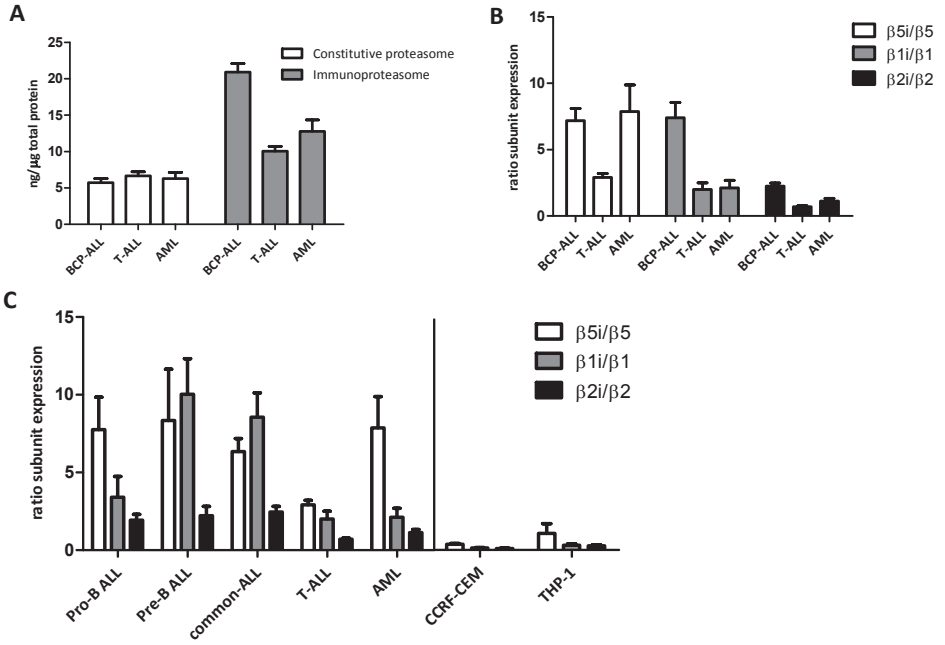
Table S2. Correlations between drug-sensitivity (in LC_{50} values) and subunit ratios obtained using Pro-CISE for ALL and AML

	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)
CFZ	-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)
BTZ	-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)

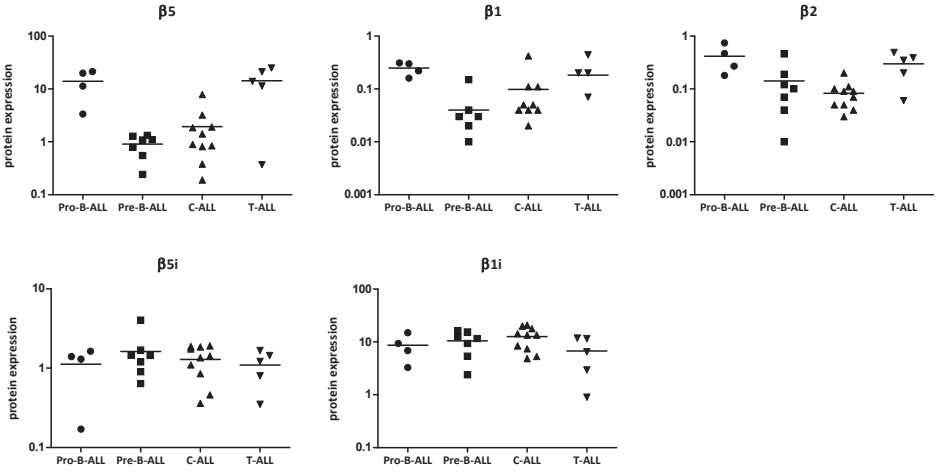
SUPPLEMENTAL FIGURES



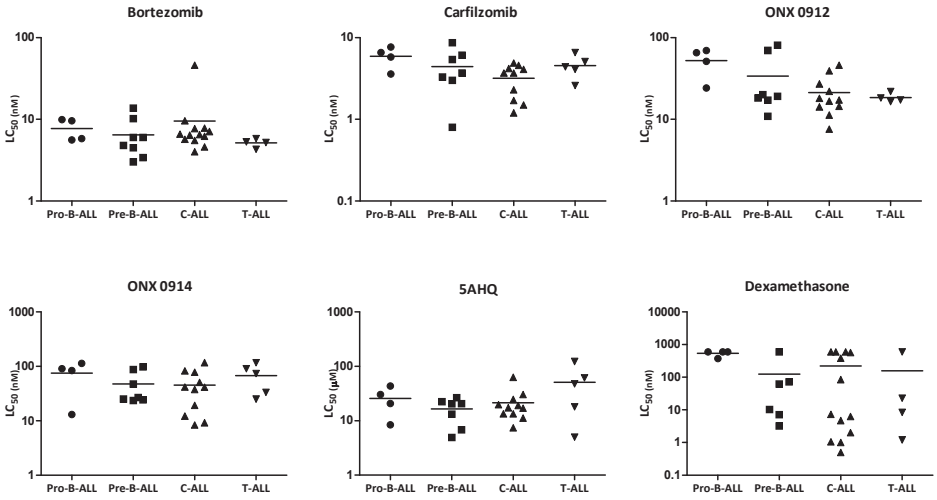
Supplementary Figure S1. Proteasome protein subunit expression of ALL and AML patient samples determined by ProCISE. Subunit protein expression of ALL and AML patient samples is depicted as ng subunit/µg total protein of **(A)** ß5, **(B)** ß1,**(C)** ß2, **(D)** ß5i,**(E)** ß1i, and **(F)** ß2i, determined by proteasome constitutive immuno subunit ELISA. The line represents the mean.



Supplementary 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 subunitswithin 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 THP-1. Error bars represent standard error of the mean.



Supplementary Figure S3. Proteasome subunit expression of ALL subgroups. ALL patient samples divided into subgroups by immunophenotype. Protein expression of ß5, ß1, ß2, ß5i, and ß1i was determined by Western blotting and normalized on actin as loading control and to the subunit expression of the CEM cell line as control between blots. The line represents the mean.



Supplementary Figure S4. Drug-sensitivity of ALL subgroups. LC_{50} concentrations of BTZ, DEX, CFZ, 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.