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CHAPTER

8

Ratios of immunoproteasome over constitutive proteasome expression are an indicator for sensitivity to bortezomib-containing reinduction chemotherapy in pediatric relapsed acute lymphocytic leukemia and acute myeloid leukemia

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Manuscript in preparation

ABSTRACT

Bortezomib, a proteasome inhibitor, is in Phase 3 clinical trials for the treatment of pediatric acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). For optimal personalized treatment, identification of bortezomib-responders is of clinical interest. Here, we investigated whether ratios of immunoproteasome to constitutive proteasome protein correlate with clinical outcome in first relapsed and refractory pediatric acute leukemia. Patients were enrolled in two Children's Oncology Group (COG) phase 2 clinical trials of bortezomib with reinduction chemotherapy for pediatric acute ALL (COG-AALL07P1) and pediatric AML (COG-AAML07P1). Protein expression of proteasome subunits was examined in 58 acute leukemia patient samples (ALL n=46, AML n=12) obtained before bortezomib-containing reinduction therapy. AML myeloblasts showed significantly 3-fold higher constitutive $\beta 5$ and $\beta 1$ subunit expression in AML cells than ALL lymphoblasts ($P=0.004$ and $P=0.026$). Ratios of both $\beta 5i/\beta 5$ and $\beta 1i/\beta 1$ subunit expression were significantly 2.5-fold higher in ALL cells than in AML cells ($P=0.013$ and $P=0.003$). Upon stratification of patients by attainment of complete remission (CR) following induction, ratios of both $\beta 5i/\beta 5$ and $\beta 1i/\beta 1$ were significantly 2-fold higher in patients that attained a CR (N=37) compared to patients that did not (N=21; $P=0.019$ for $\beta 5i/\beta 5$, $P=0.022$ for $\beta 1i/\beta 1$). In addition to protein expression ratios, increased ratios of pre-treatment proteasome subunit-specific catalytic activity of $\beta 5i/\beta 5$ were observed in patients that reached CR (N=10) compared to those that did not (N=14; $P=0.056$). Together, these results suggest that higher ratios of immuno/constitutive proteasome in pretreatment acute leukemia can serve as a novel putative predictor for the clinical response to bortezomib-containing treatment.

INTRODUCTION

Survival of pediatric patients with leukemia has greatly improved in recent decades. However, 20-40% of patients relapse and outcome after relapse remains poor, with remission rates as low as 10–40% for poor prognostic subtypes^{1,2}. There is a need for improvement in therapeutic options for these patients. Based on the clinical activity in adult hematologic malignancies such as multiple myeloma (MM)³ and mantle cell lymphoma⁴, the proteasome inhibitor bortezomib is being tested in pediatric leukemia. The proteasome inhibitor bortezomib is thought to block NF- κ B chemotherapy-induced pro-survival pathways in the leukemic blasts by abrogating the proteasomal degradation of NF- κ B's natural inhibitor, I κ B δ . Bortezomib has modest single-agent activity in children⁶ and adults⁷⁻⁹. In line with leukemic cell lines where bortezomib was shown to interact in an additive or synergistic way when combined with traditional drugs including glucocorticoids¹⁰, phase 1/2 studies combining bortezomib with conventional chemotherapeutics showed promising clinical activity in adult AML patients¹¹⁻¹³ and pediatric ALL patients^{14,15}. In contrast, bortezomib combined with reinduction chemotherapy was tolerable in relapsed pediatric AML, but bortezomib did not improve complete remission (CR) rate and overall survival of this patient cohort¹⁶. This study closed prematurely due to failure to meet efficacy criteria and illustrates that this treatment strategy warrants improvement in AML. Despite the encouraging therapeutic results of bortezomib presented so far, primary or acquired resistance to bortezomib may limit its efficacy¹⁷. Hence, identifying patients that

respond to bortezomib-containing therapy has clinical significance. Recently, we reported that higher ratios of immunoproteasome to constitutive proteasome protein expression in pediatric ALL and AML leukemia cells at diagnosis were an accountable factor for *ex vivo* sensitivity to bortezomib and next generation proteasome inhibitors¹⁸. The importance of subunit assembly for proteasome inhibitor sensitivity was further investigated in a mechanistic cell line study revealing that interferon- γ -induced upregulation of immunoproteasome subunit expression, and concomitant downregulation of constitutive subunit expression, markedly resensitized bortezomib-resistant cells towards proteasome inhibitors. $\beta 5i$ was the most important subunit involved in this process¹⁹. Here, we explored whether ratios of immunoproteasome to constitutive proteasome protein expression correlated with response to bortezomib. The group tested were pediatric patients with first relapsed or refractory acute leukemia patients enrolled in two Children's Oncology Group (COG) Phase 2 clinical trials of bortezomib combined with re-induction chemotherapy for pediatric ALL and pediatric AML. In addition, a feasibility study of bortezomib-induced changes in NF- κ B activity was performed in the same patients. Interestingly, a decrease in NF- κ B activity was observed 24h after the first dose of bortezomib, but this decrease was not associated with attainment of CR. Rather, in line with our previous work, we found that higher ratios of both immune-/constitutive proteasome subunit expression and catalytic activity was associated with CR in patients receiving bortezomib-containing reinduction therapy.

MATERIALS AND METHODS

PATIENTS

Pre-treatment, snap-frozen material was obtained from 93 patients enrolled in either the ALL (N=70) or AML (N=23) clinical trials between 2009-2013. Since no differences in overall survival between the two randomization arms in AAML07P1 were observed¹⁶, no distinction was made between AML patients receiving etoposide or idarubicin. For logistic reasons, the collected samples did not undergo a blast purification procedure, and had therefore variable blast counts. Only those samples with blast percentages >20% were included in the analyses, which is commonly used as general threshold of blast percentage for the diagnosis of acute leukemia²⁰. Of 61 patient samples available for proteasome subunit analysis, 3 samples were omitted due to very low blast percentages (0-11%), leaving 58 patient samples (46 ALL and 12 AML) for proteasome subunit expression, and 24 patient samples (14 ALL and 10 AML) for proteasome subunit catalytic activity. Of 79 peripheral blood samples available for NF- κ B activity measurements, 31 were excluded due to very low blast percentages, leaving 48 patient samples (36 ALL and 12 AML) evaluable for analysis.

PROTEIN EXPRESSION/WESTERN BLOTTING

For proteasome expression studies, bone marrow samples were used with blast percentages ranging from 21-99% for ALL (median = 85%) and from 22-77% for AML (median = 60%). Antibodies to proteasome subunits $\beta 1$, $\beta 2$, $\beta 5$, $\beta 1i$, and $\beta 5i$ were purchased from Enzo Life Sciences (Farmingdale, NY, USA), and the IRDye infrared-labeled secondary antibodies were from LI-COR Biosciences (Lincoln, NE, USA). In addition, anti-actin (clone C4) was purchased from Millipore (Temecula, CA, USA). Protein expression levels of constitutive proteasome subunits $\beta 5$ and $\beta 1$, and immunoproteasome subunits $\beta 5i$ and $\beta 1i$ were determined by

Western blot analysis, as previously described²¹. Protein bands were quantified by Odyssey software, corrected for background, and normalized with β -actin. Subunit expression between patient samples on different gels were normalized using subunit expression in the leukemic T-ALL cell line CCRF-CEM²¹. As such, Western blot data depicts relative quantifications of subunit expression.

SUBUNIT-SPECIFIC $\beta 5$, AND $\beta 5i$ PROTEASOME CATALYTIC ACTIVITIES

Subunit specific proteasome catalytic activity of $\beta 5$ and $\beta 5i$ were measured using fluorogenic substrates²² as previously described¹⁹, in cell extracts of 14 ALL and 10 AML pre-treatment samples.

ACTIVE p65 NF- κ B BY ELISA

Peripheral blood mononuclear cells were isolated from whole blood of patients (N=79) prior to bortezomib treatment and at 6h and 24h after the first bortezomib dose. For NF- κ B activity experiments, blast percentage ranged from 21-93% (median 53.5%). Specifically for ALL (N=36), blast percentages ranged from 21-93% (median 53.5%), and for AML (N=12) blast percentage ranged from 22-85% (median 52%). Nuclear lysates were prepared and active p65 NF- κ B was determined by ELISA as previously described⁶.

STATISTICS

Correlations of subunit expression or catalytic activity with initial response to therapy were calculated by determining Spearman correlation coefficients. Statistical significance between groups was determined by Mann-Whitney U test and was achieved when $P < 0.05$ (two-tailed). All statistical analyses were performed using IBM-SPSS (version 20.0).

RESULTS

PROTEASOME SUBUNIT EXPRESSION IN RELAPSED CHILDHOOD ACUTE LEUKEMIA SAMPLES

Similar to previous results in patient samples at initial diagnosis¹⁸, in this cohort of 46 relapsed ALL and 12 AML patients, pre-treatment AML blasts had significant 3-fold higher constitutive $\beta 5$ and $\beta 1$ subunit expression than ALL blasts ($P=0.004$ and $P=0.026$, respectively; Figure 1A-D; Table S1). Whereas this could be accounted for by a significantly higher blast percentage in ALL cells (median: 85%) than in AML cells (median: 60%) ($P=0.001$), no significant differences were observed for $\beta 5i$ and $\beta 1i$ immunoproteasome subunit expression between relapsed AML and ALL (Figure 1B-E). As a consequence, ratios of both $\beta 5i/\beta 5$ and $\beta 1i/\beta 1$ expression were significantly 2.5-fold higher in ALL patients than in AML patients ($P=0.013$ and $P=0.003$, respectively; Figure 1C-F; Table S1). In addition, $\beta 1i/\beta 1$ expression ratios were significantly 4-fold lower in T-ALL (N=10) compared to pre-B ALL (N=36) ($p=0.01$), and a trend for different $\beta 5i/\beta 5$ expression ratios was observed ($P=0.1$) (supplemental Figure S1). Blast percentages did not differ between T-ALL (median 83.5%) and pre-B ALL (median 86.5%). Although there was a good correlation between proteasome subunit expression and catalytic activity ($R=0.61$ $P=0.01$), the activity of the subunits $\beta 5$, $\beta 5i$, $\beta 1i$, and the activity ratio of $\beta 5i/\beta 5$ did not differ between ALL (N=14) and AML (N=10) patients (supplemental Figure S2).

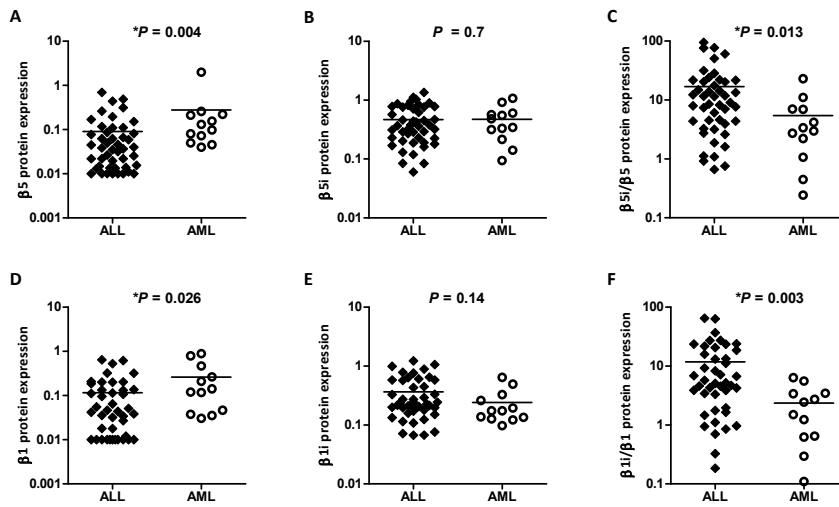


Figure 1. Proteasome subunit protein expression in relapsed childhood ALL and AML. Comparison of (A) constitutive subunit $\beta 5$, (B) immunoproteasome subunit $\beta 5i$, (C) the ratio of paired subunits $\beta 5i/\beta 5$, (D) constitutive subunit $\beta 1$, (E) immunoproteasome subunit $\beta 1i$, and (F) the ratio of paired subunits $\beta 1i/\beta 1$, within each patient sample. Protein expression was assessed by Western blotting and expressed as relative quantifications of subunit expression (ratio proteasome subunit/ β -actin based on loading of 15 μ g total protein, normalized to CEM). The line denotes the mean for ALL (N=46) and AML (N=12) patient samples.

CORRELATES OF SUBUNIT PROTEIN EXPRESSION WITH RESPONSE TO RE-INDUCTION CHEMOTHERAPY

Next, we determined differences in proteasome protein subunit expression between patients that achieved CR after the first reinduction cycle versus patients that did not achieve CR at this time point. Upon stratification of all patients, regardless of the leukemia subtype, ratios of both $\beta 5i/\beta 5$ and $\beta 1i/\beta 1$ were significantly 2-fold higher in patients who reached CR (n=37) compared to patients who did not reach CR (n=21) ($P=0.019$ for $\beta 5i/\beta 5$, $P=0.022$ for $\beta 1i/\beta 1$; Figure 2A-B). Individual subunit expression showed a significant 2-fold higher $\beta 5$ expression ($P=0.03$) in patients who reached CR versus patients who did not reach CR, while other subunits were not differently expressed (supplemental Figure S2). In addition to protein level ratios, $\beta 5i/\beta 5$ subunit activity ratios were higher in patients who reached CR (N=10) compared to patients who did not reach CR (N=14) ($P=0.056$; Figure 2C). No differences were noted in individual subunit catalytic activities. Stratifying patients into AML and ALL subtypes, 34 (74%) of the ALL patients achieved CR, whereas 12 (26%) did not. For the AML patients, 3 (25%) achieved CR, whereas 9 (75%) did not. Although similar trends in differences in $\beta 5i/\beta 5$ and $\beta 1i/\beta 1$ ratios as in the total group were seen, significance was lost due to limited numbers per group (supplemental Figure S3; Table S1).

THE EFFECT OF BORTEZOMIB TREATMENT ON NF- κ B ACTIVITY

Furthermore, we explored the predictive value of NF- κ B activity. Therefore, NF- κ B activity was evaluated in peripheral blood of 36 ALL patients (26 pre-B ALL and 10 T-ALL) and 12 AML patients before the first bortezomib gift, 6 hours after, and 24 hours post-treatment.

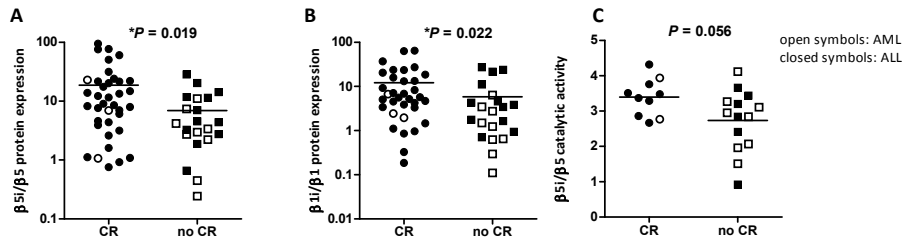


Figure 2. Ratio proteasome subunit protein expression in relapsed childhood acute leukemia patients who achieved complete remission versus patients who did not. Ratios of immunoproteasome/constitutive proteasome protein expression determined by Western blot analysis comparing; (A) $\beta 5i/\beta 5$ ratios and (B) $\beta 1i/\beta 1$ ratios between patients who achieved complete remission (CR, N=37) after bortezomib-containing re-induction therapy versus patients who did not achieve CR (no CR, N=21). Protein expression is expressed as relative quantifications of subunit expression (ratio proteasome subunit/ β -actin based on loading of 15 μ g total protein, normalized to CEM). (C) Proteasome $\beta 5i/\beta 5$ catalytic activity ratios in patients who achieved complete remission (N=10) versus patients who did not (N=14). The open symbols represent AML patients, the closed symbols ALL patients. The line denotes the mean.

NF- κ B activity was significantly higher in AML versus ALL patients before reinduction treatment ($P=0.004$, Figure 4A). Twenty-four hours after bortezomib treatment, pre-B ALL patients who attained a CR (N=16) displayed a significant decrease in NF- κ B activity compared to pre-treatment levels ($P=0.006$, Figure 4B). In contrast, pre-B ALL patients who did not attain a complete remission (n=10) after the first bortezomib cycle showed unaltered NF- κ B activity after bortezomib treatment, similar to T-ALL patients (N=8) who achieved CR. Overall, changes in NF- κ B activity during treatment with bortezomib-containing chemotherapy were not associated with post-induction CR.

DISCUSSION

The current research is the first to describe the relation between *in vivo* sensitivity to bortezomib and proteasome subunit composition in a large series of pediatric acute leukemia patients. Bortezomib, a proteasome inhibitor with FDA approval for MM, follicular and mantle cell non-Hodgkin lymphoma, is currently being explored in pediatric acute leukemia treatment. However, not all MM patients respond to bortezomib treatment¹⁷, a notion that could limit the use of bortezomib in pediatric leukemia. In order to prevent unnecessary treatment and toxicity, there is an urgent need for predictive factors for response. Our recent *ex vivo* study suggested that ratios of immunoproteasome subunits to constitutive subunits correlated with response of acute leukemia cells to proteasome inhibitors¹⁸. The rationale for this study was based on *in vitro* results revealing that bortezomib-resistant hematologic cells displayed upregulated constitutive proteasome subunit protein expression, and concomitant downregulation of immunoproteasome subunit levels compared to bortezomib-sensitive parental cells^{21,23}. These bortezomib-resistant cell lines were characterized by mutations in the *PSMB5* gene encoding the $\beta 5$ subunit. To date, no *PSMB5* mutations have been found in patients clinically resistant to bortezomib²⁴⁻²⁶. In the current study, no *PSMB5*-associated mutations in exon 2 of the gene were identified at either end-of induction (N=15) or relapse

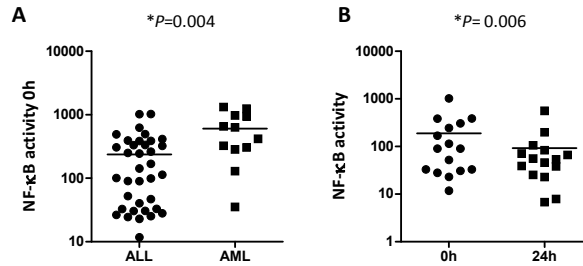


Figure 3. Baseline NF-κB activity between ALL and AML patients and impact of bortezomib treatment. NF-κB activity measurements determined by p65-ELISA in peripheral blood of acute leukemia patients prior to the first bortezomib administration, comparing (A) NF-κB activity of ALL (N=36) patients versus AML (N=12) patients, and (B) NF-κB activity prior to treatment compared to 24 hours after bortezomib therapy in pre-B ALL patients (N=16). The line denotes the mean.

(N=3) samples (data not shown). This could imply that, in acute leukemia, $\beta 5$ mutations make a minor contribution to bortezomib resistance, and that other resistance mechanisms, such as overexpression of $\beta 5$, may play a more important role^{21,23,27}. Similar to our earlier work¹⁸, the current study shows that constitutive ($\beta 5$ and $\beta 1$) proteasome subunit expression was significantly lower in ALL vs. AML patients, whereas $\beta 1i$ and $\beta 5i$ immunoproteasome subunit expression were increased in ALL. Others have also reported that bortezomib-sensitivity relates to proteasome expression. In particular, increased *PSMB5* mRNA expression was found in a myeloma patient; this patient subsequently developed bortezomib-resistance²⁵. Moreover, bortezomib-sensitive hematologic cells harbored higher immunoproteasome expression levels compared to relatively bortezomib-resistant solid-tumor cell lines²⁸. Lastly, a higher $\beta 2/\beta 1+\beta 5$ activity ratio correlated with higher bortezomib-sensitivity in cell line panel of hematologic malignancies²⁹. In a recently conducted mechanistic study, we showed that interferon- γ -induced upregulation of immunoproteasome expression and concurrent constitutive proteasome subunit downregulation in bortezomib-resistant hematologic tumor cell lines resulted in the sensitization for proteasome inhibitor treatment¹⁹. In addition, knockdown of constitutive $\beta 5$ in the AML cell line THP1 resulted in increased sensitivity to bortezomib²³. To determine the possible predictive potential of the $\beta 5i/\beta 5$ proteasome subunit expression ratio we dichotomized the ratio data on higher or lower than the mean and calculated the predictive values. Remarkably, the positive predictive value was high (87.5%), indicating that patients with a high $\beta 5i/\beta 5$ ratio indeed have a good chance of attaining a CR. However, the negative predictive value is only 45%, suggesting that this parameter does not yet suffice for the selection of patients that will not respond to bortezomib-containing therapy. A limitation of this study would potentially relate to the variation in blast percentage between samples. However, when a selection was made of only samples with blast percentage above the median, outcome was unaltered, indicating that results are representative for an (almost) pure blast population. Beyond the impact of proteasome subunit composition for proteasome inhibitor response, we could also

evaluate NF- κ B activity in 36 ALL and 12 AML samples after the first dose of bortezomib. NF- κ B is constitutively active in the majority of ALL patients^{14,30} and AML patients³¹. Though a significant decline in NF- κ B activity was observed in pre-B-ALL patients 24h after the first bortezomib dose, this decline in NF- κ B activity did not correlate bortezomib response. In contrast, Magrangeas et al.³² found that low levels of NF- κ B associated with higher response rate to bortezomib-based induction in newly diagnosed MM. Since both canonical and non-canonical pathways contribute to total NF- κ B activity, the activation of the non-canonical pathway possibly compensates for the inhibition of the canonical pathway⁵. In this regard, a limitation to this study is therefore that the assay employed only covers the canonical p65-mediated NF- κ B activation pathway whereas the non-canonical pathway was not studied. This also holds for assessment of the mutational status of common genes within the non-canonical pathway as reported in MM patients³³. Hideshima et al³⁴ indicated that the cytotoxicity of bortezomib was not associated with NF- κ B inhibition, after they observed that bortezomib triggered NF- κ B activity via the canonical pathway in MM cell lines in patient specimen. However, their analyses covered a relatively short-term exposure to bortezomib (8-12 hours), thus the initial upregulation of NF- κ B might reflect a transient stress-response by the cells to bortezomib. These effects can also be cell type related, since the canonical and non-canonical NF- κ B activity pathways are also differentially active in different MM cell lines³⁴. A Phase 1 COG study in relapsed/refractory ALL patient cells also revealed an initial increase in NF- κ B activity 3-6 hours after bortezomib, however, this study involved only a small number of patients⁶. In the extended study presented here, we showed that 13 (37%) ALL patients had elevated NF- κ B activity 6h after bortezomib treatment, indicating that NF- κ B activity in response to bortezomib differs considerably between patients. Since percentage of blasts also varied considerably between patient samples and correlated significantly with NF- κ B activity, it may be a confounding factor, which should be accounted for in future studies by performing blast purification prior to the measurements.

In summary, cells of relapsed ALL patients had significantly higher β 5i/ β 5 and β 1i/ β 1 proteasome subunit ratio expression, and lower constitutive subunit expression compared to cells of relapsed AML patients. Interestingly, patients that achieved CR after bortezomib-containing therapy had higher expression of immunoproteasome/constitutive proteasome ratios before start of re-induction therapy compared to patients that did not achieve CR. This finding was corroborated for β 5i/ β 5 catalytic activity ratios. These results suggest that higher ratios of immuno-/constitutive proteasome in pretreatment ALL and AML cells can serve as an accountable factor for the initial clinical response to bortezomib-containing re-induction treatment in relapsed pediatric acute leukemia patients.

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SUPPLEMENTAL TABLE

Table S1. Median proteasome subunit protein expression, catalytic activity and NF-κB activity in pediatric acute leukemia patients

	Expression					Catalytic activity		
	β5 (range)	β1 (range)	β5i (range)	β1i (range)	β5i/β5 (range)	β1i/β1 (range)	β5i/β5 (range)	NF-κB activity
ALL+AML	0.05 (0.01-1.98)	0.05 (0.01-0.88)	0.36 (0.06-1.35)	0.22 (0.07-1.23)	7.5 (0.24-94.5)	4.6 (0.11-64.7)	3.2 (0.92-4.3)	256.2 (11.8-1323.7)
Complete remission	0.04 (0.01-0.69) [†]	0.04 (0.01-0.64)	0.38 (0.06-1.35)	0.27 (0.07-1.06)	9.0 (0.76-94.5) [†]	5.7 (0.18-64.7) [†]	3.4 (2.7-4.3)	273.5 (11.8-1323.7)
No complete remission	0.08 (0.01-1.98)	0.12 (0.01-0.88)	0.34 (0.09-1.08)	0.20 (0.07-1.23)	4.2 (0.24-28.5)	2.7 (0.11-27.2)	2.9 (0.92-4.1)	196 (24.5-1246.5)
ALL	0.038* (0.01-0.69)	0.043* (0.01-0.64)	0.36 (0.06-1.35)	0.23 (0.07-1.23)	8.8* (0.66-94.5)	5.2* (0.18-64.7)	3.3 (1.5-4.3)	128.1 (11.8-1025.7)*
Complete remission	0.034 (0.01-0.69)	0.042 (0.01-0.64)	0.4 (0.06-1.35)	0.27 (0.07-1.06)	10.3 (0.76-94.5)	6.7 (0.18-64.7)		253.5 (11.8-1022.2)
No complete remission	0.044 (0.01-0.49)	0.047 (0.01-0.32)	0.32 (0.13-0.82)	0.22 (0.07-1.23)	6.0 (0.66-28.5)	4.1 (0.7-27.2)		73.0 (24.5-1025.7)
AML	0.12 (0.04-2.0)	0.13 (0.03-0.88)	0.41 (0.09-1.08)	0.17 (0.1-0.64)	3.2 (0.24-23.0)	2.0 (0.11-6.4)	2.9 (0.92-4.1)	524.5 (35.3-1323.7)
Complete remission	0.046 (0.04-0.13)	0.047 (0.038-0.26)	0.32 (0.14-0.92)	0.26 (0.13-0.64)	7.0 (1.1-23.0)	3.4 (2.4-5.6)		804.1 (284.5-1323.7)
No complete remission	0.16 (0.05-2.0)	0.14 (0.03-0.88)	0.48 (0.09-1.1)	0.17 (0.1-0.49)	3 (0.24-11.1)	1.2 (0.11-6.4)		524.5 (35.3-1246.5)

* Significantly different from AML, [†]Significantly different from no complete response

Please note that Western blot data depict relative quantifications of subunit expression (ratio proteasome subunit / β-actin based on loading of 15 ug total protein, normalized to CEM)

SUPPLEMENTAL FIGURES

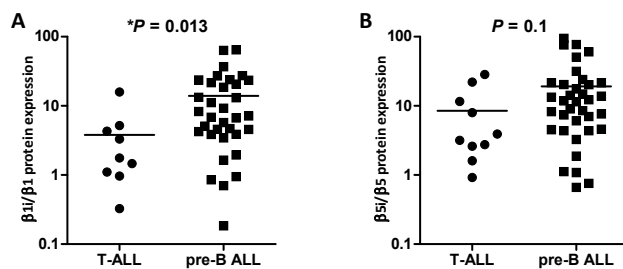


Figure S1. Ratio proteasome subunit protein expression in relapsed pediatric pre-B ALL and T-ALL patients. Ratios of immunoproteasome/constitutive proteasome protein expression determined by Western blot analysis comparing; (A) $\beta 1i/\beta 1$ ratios and (B) $\beta 5i/\beta 5$ ratios between pre-B ALL (N=36) and T-ALL (N=10) patients. The line denotes the mean.

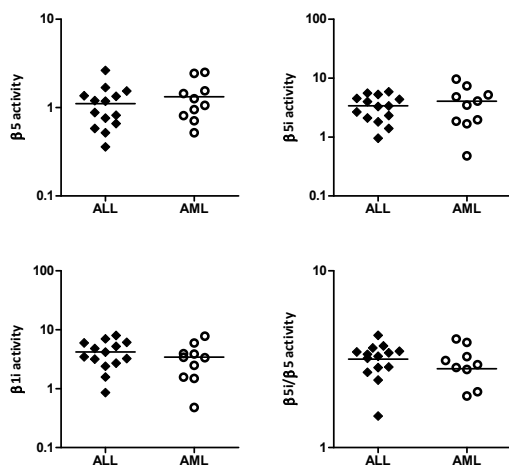


Figure S2. Proteasome subunit catalytic activity ratios between relapsed childhood ALL and AML patients. Comparison of catalytic activity of constitutive subunit $\beta 5$, immunosubunits $\beta 5i$ and $\beta 1i$, and the ratio of paired subunits $\beta 5i/\beta 5$ within each patient sample. De line denotes the mean for ALL (N=14) and AML (N=10).

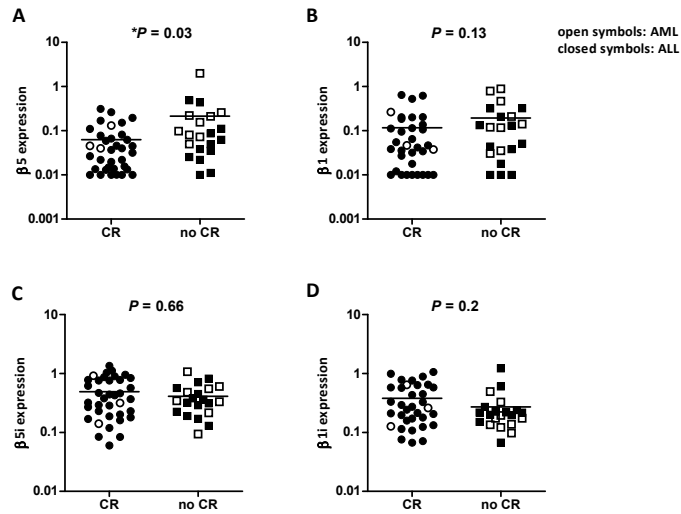


Figure S3. Individual proteasome subunit protein expression in relapsed childhood acute leukemia patients who achieved complete remission versus patients who did not. Comparison of (A) constitutive subunit $\beta 5$, (B) constitutive subunit $\beta 1$, (C) immunoproteasome subunit $\beta 5i$, (D) immunoproteasome subunit $\beta 1i$, within each patient sample. Protein expression was assessed by Western blotting and expressed as relative quantifications of subunit expression (ratio proteasome subunit/ β -actin based on loading of 15 μ g total protein, normalized to CEM). The open symbols represent AML patients, the closed symbols ALL patients. The line denotes the mean.

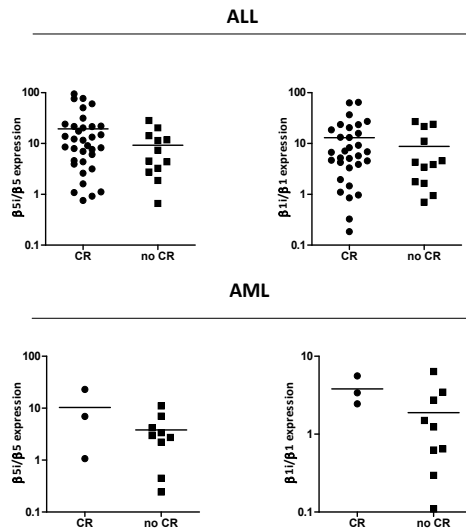


Figure S4. Ratio proteasome subunit protein expression in patients who achieved complete remission versus patients who did not, dissected by leukemia subtype. Ratios of immunoproteasome/constitutive proteasome protein expression determined by Western blot analysis after bortezomib-containing re-induction therapy versus patients who did not achieve CR. 34 ALL patients achieved CR, while 12 did not. For the AML patients, 3 achieved CR, while 9 did not. The line denotes the mean.