Chapter 4

Molecular basis of bortezomib resistance: Proteasome subunit β5 (PSMB5) gene mutation and overexpression of PSMB5 protein


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ABSTRACT

The proteasome inhibitor bortezomib is a novel anticancer drug that has shown promise in the treatment of refractory multiple myeloma. However, its clinical efficacy has been hampered by the emergence of drug resistance phenomena, the molecular basis of which remains elusive. Towards this end, we here developed high levels (45-129 fold) of acquired resistance to bortezomib in human myelomonocytic THP1 cells by exposure to stepwise increasing (2.5-200 nM) concentrations of bortezomib. Study of the molecular mechanism of bortezomib-resistance in these cells revealed (1) an Ala49Thr mutation residing in a highly conserved bortezomib-binding pocket in the proteasome β5 subunit (PSMB5) protein, (2) a dramatic overexpression (up to 60-fold) of PSMB5 protein but not of other proteasome subunits including PSMB6, PSMB7 and PSMA7, (3) high levels of cross-resistance to β5 subunit-targeted cytotoxic peptides 4A6, MG132, MG262 and ALLN, but not to a broad spectrum of chemotherapeutic drugs, (4) no marked changes in chymotrypsin-like proteasome activity, and (5) restoration of bortezomib sensitivity in bortezomib-resistant cells by siRNA-mediated silencing of PSMB5 gene expression. Collectively, these findings establish a novel mechanisms of bortezomib-resistance associated with the selective overexpression of a mutant PSMB5 protein.
INTRODUCTION

The ubiquitin proteasome system (UPS) facilitates the degradation of ubiquitin-tagged intracellular proteins many of which play a regulatory role in cell proliferation, cell survival and signaling processes. As such, proteasome inhibitors have been recognized as a new generation of chemotherapeutic agents and anti-inflammatory drugs. The boronic dipeptide bortezomib (PS341, Velcade) is the first proteasome inhibitor that has been approved for the treatment of refractory multiple myeloma. Bortezomib is a reversible inhibitor that targets primarily the β5 subunit (PSMB5) subunit/chymotrypsin-like activity of the 26S-proteasome and to a somewhat lesser extent also caspase-like activity harbored by the β1 (PSMB6) proteasome subunit. At higher concentrations, bortezomib inhibits trypsin-like proteolytic activity facilitated by β2 (PSMB7) proteasome subunits. Despite promising clinical activity, some patients with multiple myeloma failed to respond to bortezomib therapy. Moreover, the efficacy for bortezomib may differ between tumor types. Whether these observations are related to common mechanisms of drug resistance frequently seen for anti-cancer or anti-inflammatory drugs is largely unknown. However, their characterization is of key importance as it may pave the way for the overcoming of drug resistance, thereby enhancing the efficacy of this new class of proteasome-targeted drugs.

One mode of primary resistance to bortezomib is conveyed by constitutively high levels of heat shock protein 27. In the context of acquired resistance, studies aimed at delineating the mechanism of acquired resistance to the tripeptidyl aldehyde proteasome inhibitor ALLN (N-acetyl-leucyl-leucyl-norleucinal) revealed two possible molecular mechanisms; (a) enhanced cellular efflux via the multidrug resistance (MDR) transporter P-glycoprotein (Pgp; ABCC1) or multidrug resistance-related protein 1 (MRP1; ABCC1) and (b) increased detoxification via up-regulated activity of aldo-keto reductase. Recent studies showed that Pgp-overexpression conferred low levels (3-fold) of bortezomib resistance as well as to the irreversible proteasome inhibitor epoxomicin, but cells with 4-7 fold acquired resistance to epoxomicin did not show Pgp overexpression as a molecular basis of resistance. Two independent reports analyzing acquired resistance to the irreversible proteasome inhibitor NLVS (NIP-Leu3-vinyl sulfone) in mouse EL4 thymoma cells showed loss of proteasome function that was compensated by over-expression of another protease activity, tripeptidylpeptidase II. However, a more detailed characterization of these NVLS-resistant EL4 cells revealed that residual proteasome activity was sufficient to sustain cell function and viability so that the actual modality of resistance in these cells remains to be elucidated.

Altogether, given the lack of a profound understanding of the mechanisms of acquired resistance to bortezomib in mammalian cells, we set out to characterize human monocytic/macrophage cells with high levels of acquired bortezomib-resistance after in vitro
selection using a protocol of stepwise increase in bortezomib concentrations. Our findings establish that bortezomib resistance is not conferred by increased expression of MDR efflux pumps. Rather, qualitative and quantitative alterations were observed at the level of the β5 proteasome subunit; these included a PSMB5 gene mutation resulting in a substitution of a crucial amino acid (Ala49Thr) in the bortezomib-binding pocket of the β5 subunit. These bortezomib-resistant cells highly overexpressed this mutant β5 subunit and displayed a marked cross-resistance to β5-targeted cytotoxic peptides but not to other classes of therapeutic drugs. Hence, these findings establish a novel modality or bortezomib resistance associated with the selective overexpression of a structurally altered β5 subunit.

METHODS

Cell culture and development of BTZ-resistant cell lines

Human monocytic/macrophage THP1 cells (ATTC, Manassas, VA) were kept as a suspension culture in RPMI-1640 medium supplemented with 5% fetal calf serum, 20 mM HEPES, 2 mM glutamine and 100 µg/ml penicillin/streptomycin at 5% CO2 and 37°C. Cell cultures were seeded at a density of 3 x 10^5 cells/ml and refreshed twice weekly. Bortezomib (BTZ)-resistant THP1 cell lines were obtained by stepwise increasing extracellular concentrations of bortezomib over a period of 6 months, starting at a concentration of 2.5 nM (IC50 concentration: 3.3 nM) up to a concentration of 200 nM bortezomib. During this process, cultures were isolated that were grown in the presence of 30 nM (THP/BTZ30), 50 nM (THP1/BTZ50), 100 nM (THP1/BTZ100), and 200 nM bortezomib (THP1/BTZ200). All bortezomib-resistant cell lines retained parental doubling times (28 ± 3 hours) and morphology, and also retained their typical capacity to differentiate into adherent macrophage-like cells upon exposure to phorbol myristate acetate (PMA). To investigate the stability of the resistance phenotype, an aliquot of THP1/BTZ100 cells was cultured in the absence of bortezomib for a period of up to 6 months. These cells will be further designated as THP1/BTZ (-100) cells.

Proteasome activity in cell lysates

Chymotrypsin-like, trypsin-like and caspase-like proteolytic activities of the proteasome were measured in freshly prepared cell lysates as described previously with some minor modifications to the protocol. In brief, a total of 5 x 10^6 untreated or bortezomib-exposed THP1 cells were washed 3 times with ice-cold PBS and spun down by centrifugation (5 minutes, 250g, 4°C). Cell pellets were then resuspended in an ATP-containing lysis buffer; 10mM Tris-HCl buffer (pH 7.8) containing 5 mM ATP, 0.5 mM DTT and 5 mM MgCl2, and kept on ice for 10 minutes. For complete lysis, cells were sonicated (MSE soni-
cator, amplitude 7, for 3 x 5 seconds with 20 seconds time intervals at 4°C) followed by centrifugation (5 min, 16,000g, 4°C) to remove cell debris. The supernatant was collected and protein concentration was determined using the Bio-Rad protein assay. Fluorogenic peptide substrates to measure the chymotrypsin-like, trypsin-like and caspase-like activity were Suc-Leu-Leu-Val-Tyr-amc, Ac-Arg-Leu-Arg-amc and Z-Leu-Leu-Glu-amc, respectively, all at a 100 µM final concentration. The substrates were incubated with 20 µg of total cell protein extract in the presence or absence of specific inhibitors in a total assay volume of 200 µl. Specific inhibitors for chymotrypsin-like, caspase-like and trypsin-like activity included bortezomib (10 nM), Ac-APnLD-al (25 µM) and leupeptin (20 µM), respectively. The release of AMC was monitored online over a 2 hour time period at 37°C with 5 minute intervals. Fluorescence was measured on a Tecan Spectra Fluor apparatus (Giessen, The Netherlands) using excitation and emission wavelengths of 360 and 465 nm, respectively. Proteolytic activity was calculated from the slopes of the linear portion of the curves. All results were expressed as percentage relative to untreated THP1/WT cells (100%).

**Supplemental data**

Information on reagents and antibodies, growth inhibition assays, apoptosis assays, immunoblotting (Western blotting & native gels), gel filtration, mitochondrial membrane potential assay, gene arrays, immunofluorescence microscopy, RT-PCR/siRNA, sequence analysis and statistics can be found in the Supplemental data. The Supplemental data include five supplemental figures and legends.

**RESULTS**

**Establishment of cells with acquired resistance to bortezomib**

To explore the molecular basis of acquired resistance to bortezomib, human monocytic/macrophage THP1 cells were exposed *in vitro* over a period of 6 months to stepwise increasing concentrations of bortezomib representing approximately 1-50-fold the IC₅₀ (2.5 nM to 200 nM). THP1 cells grown in the presence of 50 nM (THP1/BTZ₅₀), 100 nM (THP1/BTZ₁₀₀) and 200 nM (THP1/BTZ₂₀₀) bortezomib were used for further characterization. Dose-response curves for bortezomib-induced cell growth inhibition revealed 45-fold (IC₅₀: 148 ± 54 nM), 79-fold (IC₅₀: 261 ± 71 nM) and 129-fold (IC₅₀: 426 ± 72 nM) levels of resistance in THP/BTZ₅₀, THP1/BTZ₁₀₀ and THP1/BTZ₂₀₀ cells, respectively, compared to wild type THP1 cells (IC₅₀: 3.3 ± 0.6 nM) (Figure 1A).
Figure 1. Emergence of acquired resistance to bortezomib and impaired bortezomib-induced accumulation of ubiquitinated proteins in bortezomib-resistant cells. (A) Dose-response curve for bortezomib-induced growth inhibition of wild-type (WT) human monocytic/macrophage THP1 cells and bortezomib (BTZ)–resistant variants THP1/BTZ50, THP1/BTZ100, and THP1/BTZ200, selected for growth in extracellular concentrations of 50 nM, 100 nM, and 200 nM bortezomib, respectively. Results depicted are the mean of 7 to 20 experiments (± SD). Drug exposure time, 72 hours. (B) Accumulation of ubiquitinated proteins in THP1/WT cells and bortezomib-resistant sublines after exposure to bortezomib. Bortezomib-resistant cells were allowed a 4-day drug washout period (control) after which they were exposed for 24 hours to their selective concentrations of bortezomib. THP1/WT cells were exposed to 10 nM BTZ for 24 hours. (C) Accumulation of ubiquitinated proteins in THP1/WT cells after 24-hour exposure to 10 to 100 nM bortezomib and for THP1/BTZ200 cells after 24-hour exposure to bortezomib concentrations beyond selective concentrations (up to 1000 nM). A representative picture of 2 separate experiments is depicted.
Cross-resistance profile of bortezomib-resistant cells to proteasome inhibitors and various anti-cancer/anti-inflammatory drugs

Bortezomib-resistant cells displayed appreciable cross-resistance to other known small (3-mer) peptide-based proteasome inhibitors (ALLN, MG132 and MG262) (Table 1), although to a lower level (6-18 fold) than for bortezomib itself. Strikingly, very high levels of cross-resistance (up to 300-fold) were observed for the 6-mer cytotoxic peptide 4A6 that exerts a specific β5 subunit-related/chymotrypsin-like proteasome inhibitory activity (R.O., Y.G.A., R.J.S., G.J., unpublished results). Of note, no appreciable levels of cross-resistance were observed for a broad spectrum of chemotherapeutic drugs with distinct mechanisms of action, for example methotrexate (folate antagonist), sulfasalazine (Inhibitor κB kinase/NFκB inhibitor), 5-fluorouracil (fluoropyrimidine antimitabolite), chloroquine (lysosomotropic drug), bleomycin (DNA-interacting agent), gefitinib (epidermal growth factor receptor tyrosine kinase inhibitor), cisplatin (DNA-intercalator), cyclosporin A (immunosuppressive drug), methyl-prednisolone (corticosteroid), geldanamycin (heat shock protein inhibitor), doxorubicin (DNA interacting drug) and mitoxantrone (topoisomerase inhibitor) (Table 1). The latter two drugs are bona fide substrates of the ATP-driven drug efflux transporters P-glycoprotein (Pgp; ABCB1), multidrug resistance-associated proteins 1-9 (MRP1-9/ABCCs) or breast cancer resistance protein (BCRP; ABCG2). The lack of cross-resistance to these drugs in bortezomib-resistant cells argues against a multidrug resistance (MDR) phenotype. This notion was further corroborated by the observation that parental mRNA levels of Pgp, MRP1-9 and BCRP were retained in bortezomib-resistant cells (data not shown) and western blot experiments (Figure S1) showing no differential expression of Pgp, MRP1-9 and BCRP in bortezomib-resistant cells versus parental THP1/WT cells. Finally, bortezomib-resistant cells lacked cross-resistance to AAF-cmk, an inhibitor of the proteolytic system tripeptidylpeptidase II. Together, these results demonstrate that cross-resistance of bortezomib resistant cells is restricted to (peptide) drugs that primarily target the proteasome’s β5 subunit.

To further characterize the bortezomib-resistant cells, and to gain insight whether multiple mechanisms of resistance may contribute to the bortezomib-resistant phenotype, microarray analysis was performed to assess differential gene expression in control THP1/WT cells versus THP1/BTZ30 cells, THP1/BTZ100 cells and its subline THP1/BTZ(-100) grown in the absence of BTZ for 6 months. The entire data set of the test series is available at the GEO database, accession number GSE11771 (https://www.ncbi.nlm.nih.gov/geo/). Preliminary evaluation of expression of genes specifically involved in drug metabolism and resistance (n=101), apoptosis (n=59) and cell cycling (n=76) showed no marked and/or robust alterations (<2 or >2-fold) that could be associated with resistance (Figure S2). Consistently, western blot analysis for specific proteins in these gene categories, e.g. Hsp27, Hsp90, XIAP and p21 showed no marked changes in bortezomib resistant cells compared to bortezomib-sensitive THP1/WT cells (Figure S3).
Strikingly, however, approximately 40% of genes representing the various proteasome subunits, in particular β-subunits, showed a markedly (≥2-fold) increased expression level (Figure S2). Only 2 proteasome subunit related genes were down-regulated in expression (≤2-fold), being the immunoproteasome subunits β5i and β1i. Of additional interest, no alterations were observed in expression levels of genes encoding ubiquitin-conjugating enzymes, ubiquitin-specific proteases or ubiquitin C-terminal hydrolases. Finally, upregulated expression of proteasome subunit genes in bortezomib-resistant cells appeared to be transient as gene expression normalized when THP1/BTZ100 cells were grown in the absence of bortezomib for 6 months (Figure S2). Together, gene array analysis further support the notion that alterations in proteasome subunit composition or function may be dominantly involved in the bortezomib resistant phenotype.

Table 1. Growth inhibitory effects of proteasome inhibitors and various anti-cancer/-inflammatory drugs for wild type (WT) and Bortezomib (BTZ)-resistant THP1 cells.

<table>
<thead>
<tr>
<th>Drug</th>
<th>THP1/WT IC50 (nM)</th>
<th>THP1/BTZ50</th>
<th>THP1/BTZ100</th>
<th>THP1/BTZ200</th>
<th>(Resistance Factor)</th>
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<tr>
<td><strong>Proteasome inhibitors</strong></td>
<td></td>
<td></td>
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<tr>
<td>Bortezomib (nM)</td>
<td>3.3 ± 0.6</td>
<td>45</td>
<td>79</td>
<td>129</td>
<td></td>
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<tr>
<td>MG132 (nM)</td>
<td>237 ± 54</td>
<td>8.1</td>
<td>11.9</td>
<td>15.8</td>
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<tr>
<td>MG262 (nM)</td>
<td>2.1 ± 0.6</td>
<td>8.3</td>
<td>10.3</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>ALLN (µM)</td>
<td>3.7 ± 0.4</td>
<td>5.8</td>
<td>10.0</td>
<td>18.1</td>
<td></td>
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<tr>
<td>4A6 (µM)</td>
<td>0.26 ± 0.06</td>
<td>44</td>
<td>117</td>
<td>287</td>
<td></td>
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<td><strong>Miscellaneous drugs</strong></td>
<td></td>
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<tr>
<td>Methotrexate (nM)</td>
<td>8.0 ± 1.4</td>
<td>0.8</td>
<td>1.0</td>
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<tr>
<td>Sulfasalazine (µM)</td>
<td>275 ± 49</td>
<td>1.3</td>
<td>0.8</td>
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<td>Chloroquine (µM)</td>
<td>56.3*</td>
<td>1.1</td>
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<tr>
<td>Cyclosporin A (µM)</td>
<td>3.9*</td>
<td>0.9</td>
<td>0.8</td>
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<tr>
<td>5-Fluorouracil (µM)</td>
<td>2.3*</td>
<td>0.5</td>
<td>0.4</td>
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<tr>
<td>Methylprednison (µM)</td>
<td>0.69*</td>
<td>1.1</td>
<td>1.0</td>
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<tr>
<td>Doxorubicin (nM)</td>
<td>18.9 ± 6.2</td>
<td>1.3</td>
<td>1.5</td>
<td></td>
<td></td>
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<tr>
<td>Mitoxantrone (nM)</td>
<td>2.5 ± 1.3</td>
<td>1.4</td>
<td>1.2</td>
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<tr>
<td>Bleomycin (µM)</td>
<td>4.9 ± 2.9</td>
<td>0.6</td>
<td>0.5</td>
<td></td>
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<tr>
<td>Cisplatin (µM)</td>
<td>0.84 ± 0.46</td>
<td>2.1</td>
<td>1.9</td>
<td></td>
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<tr>
<td>Iressa (µM)</td>
<td>10.4 ± 4.6</td>
<td>1.3</td>
<td>1.3</td>
<td></td>
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<tr>
<td>Geldanamycin (nM)</td>
<td>30.6 ± 13.1</td>
<td>1.0</td>
<td>1.1</td>
<td></td>
<td></td>
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<tr>
<td>AAF-cmk (µM)</td>
<td>7.3 ± 1.8</td>
<td>1.4</td>
<td>1.9</td>
<td>1.1</td>
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*IC50: Drug concentration resulting in 50% growth inhibition compared to control.
**Resistence factor: ratio IC50 BTZ-resistant cell line over IC50 THP1/WT cells.
* Mean of two experiments performed in duplicate.
Results depicted are the mean of at least 3-10 experiments ± S.D.
Diminished accumulation of ubiquitinated proteins in bortezomib-resistant cells

A characteristic feature of proteasome inhibition is the accumulation of ubiquitinated proteins that provokes loss of mitochondrial membrane potential and apoptosis.\textsuperscript{33,36} Consistently, marked accumulation of ubiquitinated proteins was observed in wild type cells exposed to bortezomib but not in bortezomib-resistant cells at their selective concentration of 30-200 nM (Figure 1B). However, the latter cells showed clear accumulation of ubiquitinated proteins if exposed to concentrations of bortezomib above the selective concentrations (500-1000 nM) (Figure 1C). Thus, bortezomib resistant cells have retained their capacity to accumulate ubiquitinated proteins, except that this process is initiated at markedly higher concentrations of bortezomib consistent with the resistance factor for bortezomib.

Impaired bortezomib-induced loss of mitochondrial membrane potential and induction of apoptosis in bortezomib-resistant cells

The shift in bortezomib-induced accumulation of ubiquitinated proteins in bortezomib-resistant cells paralleled the shift in bortezomib-induced loss of mitochondrial membrane potential (Figure 2A) and induction of apoptosis (Figure 2B/C). For comparison, the anti-cancer drug and topoisomerase II inhibitor etoposide (VP16) was equally effective in inducing apoptosis in wild type as well as bortezomib-resistant cells (Figure 2D). These results indicate that bortezomib-resistant cells have retained their capacity to undergo apoptosis, consistent with the lack of alterations in gene expression of apoptosis-related genes (Figure S2), but the ability to undergo apoptosis is specifically impaired for proteasome inhibitor type of drugs.

Marked overexpression of proteasome subunit β5 but not chymotrypsin-like proteasomal activity in bortezomib-resistant cells

Since the cross-resistance data (Table 1) were consistent with specific alterations at the level of proteasome subunit expression and/or catalytic activity, the expression pattern of individual β1, β2 and β5 subunits was determined in wild type and drug-resistant cells grown at their selective bortezomib concentrations. Furthermore, to explore the stability of the bortezomib-resistance phenotype, we also examined a subline of THP1/BTZ\textsuperscript{100} that was grown in the absence of bortezomib for 6 months (termed THP1/BTZ\textsuperscript{-100}). Proteasome β1 and β2 subunit expression was only slightly increased (< 2 fold), while proteasome α7 subunit expression was not significantly altered in the bortezomib-resistant cells (Figure 3A). In contrast, β5 protein levels, relatively barely detectable in parental cells, were dramatically increased (up to 60 fold) in bortezomib-resistant cells (Figure 3A). This observation was confirmed by using two different antibodies recognizing different epitopes within the β5 protein. This dramatic increase in β5 expression
Figure 2. Differential induction of apoptosis by bortezomib and VP16 in bortezomib-resistant cells. 

(A) Loss of mitochondrial membrane potential (∆ψ_m) after 24-hour exposure of THP1/WT cells and THP1/ BTZ200 cells to a concentration range of bortezomib. (B) Induction of apoptosis (annexin V-positive cells) in THP1/WT cells and THP1/BTZ200 cells after 24-hour exposure to a concentration range of bortezomib. (C) A representative flow cytometric picture of apoptosis induction (annexin V/7-AAD staining) after 24-hour incubation of THP1/WT cells and THP1/BTZ200 cells with 100 nM BTZ. (D) A representative flow cytometric picture of apoptosis induction (annexin V/7-AAD staining) after 48-hour incubation of THP1/WT cells and THP1/BTZ200 cells with 1 µM VP-16/etoposide. All results present the means (± SD) for 3 independent experiments.
Figure 3. Selective induction of proteasome β5-subunits in bortezomib-resistant cells. (A) Protein expression of proteasome β5-, β2-, and β 1-subunits and α7-subunits in wild-type and bortezomib-resistant THP1 sublines. THP1/BTZ(-100) represents a subline of THP1/BTZ100 that was grown in the absence of bortezomib for 6 months. Note: 2 different sources of β5 antibodies were used: Biomol (PW8895; Plymouth Meeting, PA) and 20S X from Novus Biologicals (Littleton, CO), the latter indicated by an asterisk (*). (B) Induction of proteasome subunits β5, β2, and β1 in relation to resistance factors to bortezomib for the selected panel of bortezomib-resistant THP1 cells. Densitometry results are presented as the mean (±SD) of 4 separate experiments. (C) Native gel electrophoresis of crude cell extracts of THP1/WT cells and THP1/BTZ100 cells subsequently analyzed for β5- and α7-subunit expression (left panels) and catalytic activity for the substrate Suc-LLVY-AMC (right panel). (D) Gel filtration of crude extracts of THP1/WT cells and THP1/BTZ100 cells via a high-performance liquid chromatography (HPLC)–linked Superdex 200 HR 10/30 column (Supelco, Bellefonte, CA). Proteins were eluted by washing the column with 20 mMTris-HCl, pH 7.5, 5 mM ATP, and 120 mM NaCl at a flow rate of 0.4 mL/min. Fractions were collected every minute and subject to Western blot analysis for β5 and α7. The column was calibrated with a mixture of purified.
was proportional to the gradually increasing concentrations of bortezomib during the stepwise selection. Notably, over a concentration range (up to 50 nM bortezomib) where bortezomib primarily inhibits chymotrypsin-like proteasome activity, β5 overexpression parallels the extent of bortezomib resistance (Figure 3B). Apart from Western blot analysis, we also explored whether or not β5 overexpression was associated with the 20S core particle of the proteasome by performing native gel electrophoresis analysis. Results depicted in Figure 3C further demonstrate an increased expression of β5 in resistant cells as compared to parental cells; but all of this increased expression was confined to the 20S proteasome complex rather than being present as unassembled "free" subunits. As a control, expression of subunit α7 remained unchanged, as did the overall proteolytic activity in both parental and resistant cells using suc-LLVY-amc as a substrate (Figure 3C). This finding was further corroborated by gel filtration experiments (Figure 3D) that demonstrated that elevated expression of β5 was observed in bortezomib-resistant cells which eluted from the column solely as a high molecular weight fraction of » 600-700 kD protein, consistent with the 20S core particle's molecular weight. In accord with the above results, α7-subunit expression was largely unaltered between parental and bortezomib-resistant cells, although its presence was not exclusively restricted to high molecular weight fractions, but also showed up in lower molecular weight fractions, possibly even as "free" subunits. Consistent with these experiments immunohistochemical analysis (Supplementary Figure S4) also confirmed the co-localization of β5 and α7 in THP1/BTZ cells.

In contrast to β5 protein levels, β5 mRNA levels in bortezomib-resistant cells were only marginally increased at the highest selection dose (Figure 4A), suggesting that the induction of β5 protein most likely is effectuated at the post-transcriptional level. As for the β5 transcript, β2 and β1 mRNA levels were only modestly elevated in cells with the highest bortezomib-resistance level (Figure 4A).

We next examined the possible role that these quantitative alterations in β5-subunit composition may bear on the various proteolytic activities of the proteasome. Proteasomal proteolytic activities represented by chymotrypsin-like activity, caspase-like activity and trypsin-like activity were measured using fluorogenic peptide substrates in cell extracts of parental cells incubated for 24 hr in the presence or absence of 10 nM bortezomib. Bortezomib-resistant cells were tested after a 4-day drug-free period (control), and after 24 hr incubation at their selective bortezomib concentration (Figure 4B). After 4 days of incubation in drug-free medium, bortezomib-resistant cells had a small (1.3-1.4 fold) increase in chymotrypsin-like activity compared to wild type cells. Under bortezomib-selective conditions (30, 100 or 200 nM), residual chymotrypsin-like activity was reduced to 8 to 10% of drug-free controls, as observed for parental cells exposed to 10 nM bortezomib. After drug wash out of bortezomib-resistant cells, caspase-like proteasome activity was significantly increased (1.8-2.3 fold, p<0.001) over parental
Figure 4. Proteasome β5-, β2-, and β1-subunit mRNA levels and proteasome subunit–related catalytic activity in wild-type and bortezomib-resistant cells. (A) mRNA levels for proteasome β5-, β2-, and β1-subunits in selected variants of bortezomib-resistant THP1 cells relative to THP1/WT cells. mRNA levels were quantified using β-glucuronidase (GUS) as reference gene and depicted relative to THP1/WT cells. (B) Chymotrypsin-like, caspase-like, and trypsin-like proteasomal activities assayed with specific fluorogenic peptide substrates in cell extracts of THP1/WT, THP1/BTZ30, THP1/BTZ100, and THP1/BTZ200 cells after a 4-day drug washout period (control) and after 24-hour incubation with 10 nM bortezomib (for THP/WT) and selective concentrations of 30 nM, 100 nM, and 200 nM bortezomib for the indicated bortezomib-resistant THP1 sublines. Controls for selective inhibition of caspase-like activity and trypsin-like activity included Ac-APnLD (25 µM) and leupeptin (20 µM), respectively. All results represent the mean (± SD) of 3 independent experiments. (C) Activity labeling of constitutive and immunoproteasome β-subunits in intact THP1/WT and selected bortezomib-resistant THP/BTZ cells using bodipyFL-Ahx3L3VS affinity probe.
Chapter 4

A

\[ \frac{\beta_5 \text{ protein}}{\alpha \text{Tubulin}} \text{ (relative to } t=0) \]

\[ 0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 7 \]

B

\[ \frac{\beta_5 \text{ mRNA}}{\beta_5 \text{ protein}} \text{ (relative to } t=0) \]

\[ 0 \quad 1 \quad 2 \quad 3 \quad 4 \quad 5 \quad 6 \quad 7 \]

C

THP1:WT

THP1:BTZ -100

THP1:BTZ 100

\[ \beta_1 \]

\[ \beta_2 \]

\[ \beta_3 \]

\[ \alpha/\beta \text{ core} \]

\[ \alpha \text{Tubulin} \]

BTZ (nM)

\[ 0 \quad 2.5 \quad 5 \quad 10 \]

\[ 0 \quad 5 \quad 100 \quad 200 \]

D

WT

BTZ -100

BTZ 200

\[ \text{Fold induction relative to WT} \]

\[ 0 \quad 2.5 \quad 5 \quad 10 \]

\[ 0 \quad 2.5 \quad 5 \quad 10 \]

\[ 0 \quad 2.5 \quad 5 \quad 10 \]

E

\[ \frac{\beta_5 \text{ protein}}{\alpha \text{Tubulin}} \text{ (relative to } t=0) \]

\[ 0 \quad 4 \quad 8 \quad 12 \quad 16 \quad 24 \]

\[ \beta_5 \text{ protein} \text{ (relative to } t=0) \]

\[ 0 \quad 4 \quad 8 \quad 12 \quad 16 \quad 24 \]

\[ \beta_5 \text{ mRNA} \text{ (relative to } t=0) \]

\[ 0 \quad 4 \quad 8 \quad 12 \quad 16 \quad 24 \]
In the presence of bortezomib, caspase-like activity was reduced to 26-35% of wild type levels. Finally, basal trypsin-like activity was 1.4-1.7-fold (p<0.01) increased in bortezomib-resistant cells as compared to wild type cells. In the presence of bortezomib, trypsin-like activity was stimulated by another 20-40% both in bortezomib-resistant cell lines and wild type cells (Figure 4B). These results indicate that the qualitative characteristics of proteasomal inhibition by bortezomib were retained in bortezomib resistant cells, whereas quantitatively, a marked up-regulation of β5-subunit protein did not lead to a β5-subunit related increase in chymotrypsin-like proteasome activity.

Finally, probing of active proteasome subunits was performed using the bodipyFL-Ahx3L3VS affinity labeling reagent. Labeling of constitutive β5 and its immunoproteasome counterpart β5i was readily observed in parental THP1 cells (Figure 4C), but markedly inhibited at relatively low levels of bortezomib resistance in THP1/BTZ30 cells. Interestingly, probing of active β5 and β5i was partly restored in THP1 cells with higher levels of bortezomib resistance including THP1/BTZ50 and THP1/BTZ100 cells, thus underscoring the fact that proteasome activity is retained.

**Stability of the bortezomib-resistance phenotype**

To investigate the stability of the resistance phenotype and expression of β5-subunit in the absence of bortezomib, resistant cells were transferred to bortezomib-free medium for a period of 7 days and showed a gradual decline of β5 expression to 40% of the original levels (Figure 5A); whereas, no substantial alterations were observed in β5-subunit mRNA levels (Figure 5B). Interestingly, however, Figure 3A demonstrated that β5-subunit expression was further reduced in THP1/BTZ(-100) cells that were cultured in bortezomib-free medium for 6 months, although they had retained a more than 35-fold resistance level for bortezomib (not shown). This prompted us to investigate whether β5-subunit expression could be rapidly re-induced upon bortezomib re-exposure. Indeed, when THP1/WT, THP1/BTZ(-100) and THP1/BTZ100 cells were exposed to a range
of bortezomib concentrations (1-3 x IC₅₀) for 24 hours, a marked and selective induction of β5 subunit expression was noted for THP1/BTZ (-100) cells and to a lesser extent for THP1/BTZ₁₀₀ cells (Figures 5C/D). Fine-tuning the 0-24 hour time course of β5 induction revealed that within 8-16 hr after bortezomib exposure, β5-subunit expression was markedly induced in THP1/BTZ (-100) cells (Figure 5E) and was accompanied by a 2-3 fold induction of β5 mRNA levels (Figure 5F). Collectively, these results suggest that once bortezomib-resistance has been provoked after chronic exposure, the drug resistance phenotype not only can remain dormant for long-term periods when cells are not exposed to bortezomib, it would also be rapidly revived upon re-exposure to bortezomib.

Identification of a mutation in the PSMB5 gene in THP1/BTZ cells

Given the stable and long-term retention of the bortezomib resistance phenotype, along with the dominant involvement of the β5 subunit, we explored whether or not a genetic alteration in the PSMB5 gene could have contributed to the onset of acquired resistance to bortezomib. To this end, we sequenced part of exon 2 of the PSMB5 gene that encodes for the highly conserved binding pocket region for proteasome inhibitors

![Sequence of PSMB5 gene](image)

Figure 6. Analysis of PSMB5 gene mutations in bortezomib-resistant THP1 cells. Sequencing of PSMB5 gene exon 2 in THP1/WT cells and various BTZ-resistant sublines: THP1/BTZ₇, THP1/BTZ₃₀, THP1/BTZ₁₀₀, and THP1/BTZ₁₀₀₀. Depicted is the single nucleotide shift (G3A) at nucleotide position 322 in THP1/BTZ₃₀, THP1/BTZ₁₀₀, and THP1/BTZ₁₀₀₀ along with the corresponding change in a single amino acid substitution (Ala⁴⁹Thr) within the mature PSMB5/β5 protein.
within the β5 protein. In THP1 cells, selected for a very low level of acquired resistance to bortezomib, that is 7 nM (THP1/BTZ), no genetic alterations could be observed (Figure 6). However, in THP1 cells displaying higher levels of bortezomib resistance including THP1/BTZ30 and THP1/BTZ100 cells, a single G to A nucleotide shift was identified at

![Figure 7](image)

**Figure 7.** SiRNA-mediated silencing of proteasome β5-subunit induction restores bortezomib sensitivity and induces apoptosis in bortezomib-resistant cells. (A) β5-siRNA–induced silencing of β5-protein expression in THP1/BTZ-100 and THP1/BTZ200 cells after 24-hour preincubation with transfection medium (DF2), control siRNA constructs (nontarget/GAPDH), and a β5-specific siRNA construct (transduction efficiency: > 85% based on siGlo+ cells by flow cytometry). After transfection, cells were incubated (24-48 hours) with the indicated concentrations of bortezomib. Protein expressions of α/β-core and β-actin are shown as controls. A representative picture of 3 separate experiments is depicted. (B) Bortezomib-induced apoptosis in THP1/WT, THP1/BTZ(-100), and THP1/BTZ200 cells after siRNA-induced prevention of induction of β5-subunit expression as described in panel A. Bortezomib exposure time: 24 hours. Bortezomib concentrations used: 25 nM for THP1/WT and THP1/BTZ(-100) cells. Means of 4 separate experiments (± SD) are shown. *P<.01. (C) Bortezomib-induced cell growth inhibition of THP1/BTZ(-100) cells after prevention of induction of β5-subunit expression by siRNA silencing as described in panel A and 24-hour exposure to 25 nM bortezomib. Means of 3 separate experiments (± SD) are shown. *P < .01.
position 322 of the PSMB5 gene. In the mature and functional β5-subunit protein, this mutation introduces an Ala to Thr substitution at amino acid 49 (Figure 6). We further established that the Ala49Thr mutation was retained in THP1/BTZ (-100) cells (Figure 6). Based on the fact that Ala49 resides in the highly conserved substrate/inhibitor binding domain of the β5 subunit, this homozygous mutation is likely to contribute to the bortezomib resistance phenotype.

siRNA-dependent silencing of β5 induction in bortezomib resistant cells reverses bortezomib sensitivity and induces apoptosis

Finally, based on data in Figure 5, we determined whether prevention of β5-subunit induction would lead to restoration of bortezomib sensitivity. To this end, the consequences of siRNA-based silencing of β5 expression were explored in THP1/WT, THP1/BTZ (-100) and THP1/BTZ200 cells. In THP1/BTZ (-100) cells, the β5-siRNA-dependent prevention of β5-subunit up-regulation after bortezomib exposure (Figure 7A) was accompanied by both a significantly higher fraction of apoptotic cells (Figure 7B) as well as increased bortezomib sensitivity (Figure 7C). For THP1/BTZ200 cells with constitutively high levels of β5 expression (Figure 3A), β5 siRNA-dependent silencing also repressed β5 expression (Figure 7A), but not to a level that compromised bortezomib resistance (results not shown). Collectively, these results further underscore the involvement of proteasome β5 subunit overexpression in conferring bortezomib resistance and provide a targeted strategy to reverse bortezomib resistance.

DISCUSSION

This study provides several lines of evidence for the proteasome involvement, that of the β5 subunit in particular, in the acquisition of bortezomib-resistance in THP1 cells; (1) A mutation in the PSMB5 gene involving an Ala49Thr substitution in the highly conserved substrate/inhibitor binding domain of the β5 subunit, (2) Selective overexpression of the mutant β5 subunit protein paralleled bortezomib-resistance levels in THP1/BTZ cells, (3) Rapid and marked induction of β5 in THP1/BTZ (-100) cells after exposure to bortezomib, (4) siRNA-guided silencing of β5 subunit gene expression restored bortezomib sensitivity and induced apoptosis, and (5) Bortezomib-resistant cells displayed marked cross-resistance to other peptide-based proteasome inhibitors, in particular those that specifically target the β5 subunit.

Crystallography data from yeast and mammalian proteasomes indicated that Ala49 is implicated in the efficient binding of bortezomib in the substrate/inhibitor binding pocket of the β5 subunit. Moreover, Ala49 is a highly conserved residue among many prokaryotic and mammalian species. As such, substitution of Ala49 for Thr containing
a neutral polar side chain is likely to have consequences for efficiency of (reversible) binding of bortezomib and other peptide-based proteasome inhibitors. Prototypically this may be exemplified by the 6-mer peptide 4A6 that selectively targets β5 (but not subunits β1 and β2) for which up to more than 250 fold level of cross- resistance was observed in the bortezomib resistant cells. The finding that cross-resistance levels for ALLN, MG132 and MG262 were lower than the resistance factors for bortezomib may be consistent with the fact that these proteasome inhibitors have additional targets apart from β5, β2 and β1 proteasome subunits, for example lysosomal proteases, which may not be affected in bortezomib-resistant cells 9,15,20,37. The potential impact of Ala49 mutations in conferring bortezomib resistance may be further supported by recent preliminary observations by Lu et al 40 showing the same PSMB5 mutation in bortezomib-resistant human lymphoblastic Jurkat T-cells.

In keeping with the notion of a prominent role of the β5 proteasome subunit in conferring high levels of resistance to bortezomib, other types of molecular mechanisms previously reported to influence bortezomib activity did not seem to apply for bortezomib resistant THP1 cells. No evidence for extensive metabolism (oxidative deboronation) of bortezomib by cytochrome P450 reactions 41 could be obtained from gene array analysis of CytP450 enzymes. A putative role for MDR-proteins in bortezomib resistant cells could be ruled out both by the lack of cross-resistance to prototypical MDR substrates (doxorubicin and mitoxantrone) and the lack of up-regulation of MDR proteins typically involved in drug resistance. Consistently, Minderman et al 28 and our laboratory (R.O, R.J.S., G.J. unpublished observations) showed that low levels (< 3-fold) of resistance to bortezomib were detected only in mammalian cells expressing high levels of Pgp, and not by other MDR drug efflux transporters such as MRPI-6 or BCRP. In fact, none of these MDR transporters were found to be overexpressed in bortezomib-resistant THP1 cells. Furthermore, up-regulation of another proteolytic system, tripeptidylpeptidase II, to compensate for proteasome inhibition by irreversible proteasome inhibitors 30-32, was ruled out based on the lack of cross-resistance to the TPP II inhibitor, AAF-cmk 33. Finally, potential loss of bortezomib activity after short drug exposures due to constitutive or transient induction of heat shock proteins (Hsp’s) 24,42 was excluded in the present chronic exposure model as judged from the lack of cross-resistance to the potent Hsp inhibitor geldanamycin 43 and unaltered hsp27 expression in bortezomib-resistant cells (Figure S3).

Up-regulation of target enzymes is a common mode of resistance to several types of chemotherapeutic drugs 44,45. In this context, effects on proteasomal β5 subunits as the primary target for bortezomib may not be unexpected. In fact, some recent studies revealed an overall induction of proteasome β-subunits (β5, β2 and β1) as an initial adaptive response to low dose bortezomib exposure 20,21,37. Results from the present study indicate that in cells with established acquired resistance to bortezomib after
chronic exposure to stepwise increasing doses of bortezomib, up-regulation of a mutant β5 protein serves as a compensatory mechanism to retain sufficient chymotrypsin-like activity. An enhanced expression of 20S proteasome core particle-associated β5 in drug-resistant cells (Figure 3C/D) may be consistent with this notion. Also data from proteasome activity labeling experiments (Figure 4C) indicate that at relatively low levels of bortezomib resistance (THP1/BTZ30) the bodipyFL-Ahx3L3VS probe showed attenuated binding/labeling to the mutant β5 subunit, but upon further up-regulation of mutated protein in THP1/BTZ50 and THP1/BTZ100 cells, labeling with the probe can increase concomitantly. It is not readily clear whether the up-regulation of constitutive β5 in bortezomib-resistant cells (Figure 3A,C,D) is accompanied by a concomitant decrease in immunoproteasome β5 expression as suggested by gene array data (Figure S2). This status may be normalized to control levels in the absence of bortezomib, as illustrated for THP1/BTZ (-100) cells (Figure 3A), but rapidly restored upon re-challenging with bortezomib (Figure 5C). The up-regulation of β5 was most pronounced in cells exposed to bortezomib concentrations up to 50 nM, a concentration range at which primarily β5-associated chymotrypsin-like proteasome activity, and to a lesser extent also β1-associated caspase-like activity, will be essentially abolished. Consistently, beyond concentrations of 50 nM bortezomib, induction of β5 expression is leveling off as from this point inhibition of proteasome subunits β2-associated trypsin-like activity will be initiated 16,17.

The present finding that the marked overexpression of β5 protein in bortezomib-resistant cells is not paralleled by induction of β5 mRNA levels points to a post-transcriptional regulatory mechanism. The nature of possible post-transcriptional effects in bortezomib-resistant cells is presently unclear but may possibly include alterations in proteasome homeostasis facilitated by an autoregulatory mechanism that mediates the differential polyubiquitination and degradation of multiple proteasome subunits, including β5 46. Hence, impaired polyubiquitination under bortezomib selective pressure (Figure 1B) may differentially attenuate β5 degradation to yield a significant increase in β5 protein levels.

Under bortezomib selective conditions, THP1/BTZ cells did not display any accumulation of ubiquitinated proteins (Figure 1B) or induction of stress-induced proteins (Figure S3), prototypical for the action of proteasome inhibitors. Beyond the effect of the mutant β5 protein, as well as its overexpression, it may be anticipated that the up to 2-fold elevated bortezomib-stimulated trypsin-like activity (Figure 4B) may compensate for the inhibition of chymotrypsin- and caspase-like activities by sustaining proteasome activity above critical catalytic levels. Consequently, such a condition alone, or in combination with activities of deubiquitinating enzymes 47, for example Poh1 48 as a possible candidate based on preliminary gene array studies (Figure S3), could also contribute
to prevent the accumulation of toxic polyubiquitinated proteins, which may otherwise trigger loss of mitochondrial membrane potential and apoptosis.

We recently obtained evidence that a similar molecular basis of selective β5 induction after chronic exposure to bortezomib as described herein for THP1 cells also applies for bortezomib-resistant variants of human T lymphocytic CCRF-CEM cells 49 (Figure S5). Interestingly, when human 8226 myeloma cells were exposed according to the same strategy, only moderate (< 5-fold) resistance levels were observed 49. These results suggest that the onset of bortezomib resistance may differ significantly between haematological tumor cell lineages. A possible lower propensity for multiple myeloma cells to acquire resistance to bortezomib may be consistent with the lack of detailed reports on this issue. Beyond this, in the case of multiple myeloma, host microenvironments may also play an important role in the efficacy of proteasome targeting 12. With the accumulating knowledge of potential mechanisms of resistance to bortezomib, more direct screening for these parameters should be subject for future clinically-directed laboratory studies. Screening for PSMB5 gene mutations in an isolated case of a bortezomib non-responsive multiple myeloma patient did not provide evidence for mutations 50. However, based on the present observation that PSMB5 gene mutations may be provoked after exposure to clinically achievable concentrations of bortezomib in the range of 7-30 nM (Figure 6) screening for PSMB5 mutations should be reconsidered. In the context of proteasome activity and proteasome subunit composition, the recent identification and validation of specific proteasome-targeted probes 37,51 may facilitate analyses on limited cell numbers.

It may be anticipated that the molecular mechanism of resistance reported in the current study specifically applies for bortezomib or other proteasome inhibitors that selectively target the β5 subunit of the proteasome. With the emergence of second generation proteasome inhibitors targeting (ir)reversibly other proteasome subunits or multiple all β-subunits 13,19,52-54, it will be important to address whether they are able to circumvent bortezomib resistance, or that similar mechanisms of resistance are operative as described herein for bortezomib. If so, this would warrant the design of strategies to prevent selective up-regulation of specific proteasome subunits and thereby enhance the therapeutic efficacy of this novel class of therapeutic agents.

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**Authorship**


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REFERENCES


**Abbreviations**

BTZ; bortezomib, ALLN; N-acetyl-Leu-Leu-norleucinal, MG132; Z-Leu-Leu-Leucinal, MG262; Z-Leu-Leu-Leu-boronate, 4A6; Ac-Thr(tBu)-His(Bzl)-Thr(Bzl)-Nle-Glu(OtBu)-Gly-Bza, Suc-LLVY-amc; Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin, AAF-cmk; Ala-Ala-Phe-chloromethylketone, Ac-APnLD-al; Ac-Ala-Pro-Nle-Asp-CHO, OtBu; (O-)tert-butyl, Bzl; Benzyl, Bza; Benzylamine, Ac; Acetyl, TPPII; tripeptidylpeptidase II, Hsp; heat shock protein, Ub; ubiquitin, MTX; methotrexate, SSZ; sulfasalazine, CsA; cyclosporine A, CHQ; chloroquine, 5-FU; 5-Fluorouracil, TMRE; tetramethylrhodamine-ethyl ester, Pgp; P-glycoprotein, MRP1; Multidrug resistance associated protein 1, BCRP; breast cancer resistance protein.

**SUPPLEMENTARY MATERIALS AND METHODS**

**Reagents**

Bortezomib (BTZ)/Velcade® was kindly provided by Millennium Pharmaceuticals (Cambridge, USA). The proteasome-inhibitors MG132 (Z-Leu-Leu-Leucinal) and MG262 (Z-Leu-Leu-Leu-boronate) were purchased from Calbiochem/ Merck (Nottingham, UK). The cytotoxic peptide 4A6 (Ac-Thr(tBu)-His(Bzl)-Thr(Bzl)-Nle-Glu(OtBu)-Gly-Bza) was synthesized as described previously. The tripeptidylpeptidase II inhibitor H-Ala-Ala-Phe-chloromethylketone was obtained from Bachem (Germany). Chloroquine, mitoxantrone, cisplatin (CDDP), geldanamycin, doxorubicin, sulfasalazine, 5-FU, NP-40, trimethylrhodamine-ethyl ester (TMRE), ALLN (N-acetyl-Leu-Leu-norleucinal) and leupeptin (Ac-Leu-Leu-Arg-al) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Bleomycin was obtained from Dagra Pharma (Diemen, The Netherlands). Methotrexate was from Pharmachemie (Haarlem, The Netherlands). Methylprednison was purchased from Pfizer (New York, NY, U.S.A.), cyclosporin A was kindly provided by Novartis (Arnhem, The Netherlands) and Iressa®/gefitinib was a gift from AstraZeneca (Zoetermeer, The Netherlands). Protease Inhibitor Cocktail (PIC) was obtained from Roche Diagnostics (Almere, The Netherlands). RPMI-1640 tissue culture medium and fetal calf serum were obtained from Gibco Chem. Co (Grand Isl., NY, U.S.A). All fluorogenic peptide substrates (Suc-Leu-Leu-Val-Tyr-amc, Ac-Arg-Leu-Arg-amc and Z-Leu-Leu-Glu-amc), the proteasome inhibitor Ac-APnLD-al and proteasome-related antibodies (β1, β2, β5, α7, α/β core were purchased from Biomol (Plymouth Meeting, PA, U.S.A.). Antibody 20S X (NB120-3330) was obtained from Novus Biologicals, Littleton, USA). Antibodies to Hsp27 (#2402) and Hsp90 (#4875) were from Cell Signaling Technologies (Danvers, MA, USA), Antibodies to XIAP (M044-3) and P21 (OP68) were from MBL, Int. Co (USA) and Calbiochem (Germany), respectively. Anti-ubiquitin antibody (sc-8017) was purchased from Santa Cruz Biotechnology (Santa Cruz, Ca, USA).
**RT-PCR/siRNA proteasome subunits**

mRNA expression levels of proteasome subunits PSMB5 (β5), PSMB6 (β1), PSMB7 (β2) and the endogenous housekeeping gene β-glucuronidase (GUS) as a reference were quantified using real-time PCR analysis (Taqman) on an ABI Prism 7700 sequence detection system (PE Applied Biosystems). All probes were labeled with 5’-FAM and 3’-BHQ1 as a reporter. Primers and probes were designed using Primer Express software (Applied Biosystems). Primers, probe combinations and concentrations used for the quantitative real-time PCR were as follows: PSMB5 forward (50 nM): CTTCAAGTTCGCCATGGA; PSMB5 reverse (300 nM): CCGTCTGGGAGGCAA TTGAA; PSMB5 probe (200 nM): TTGCAGCTGACTCCAGGC; PSMB6 forward (300 nM): AGGCATGACCAAGGAAGAGGT; PSMB6 reverse (50 nM): GAGCCATCCCCGCTCCAT; PSMB6 probe (200 nM): TGCAATTTCTGCAAATGCTTCG; PSMB7 forward (300 nM): CCGTGTTGCTCCACCAGTT; PSMB7 reverse (50 nM): GCAAATCGGCTCCAGAC; PSMB7 probe (200 nM): TTCTCTT TTGATAACTGCCGAC; GUS forward (300 nM): GAAAATATGTGGTGGAGAGCT; GUS reverse (300 nM): CCGAGGTAGATCCCTTT; GUS probe (200 nM): CCAGACCTCTCGTGAC TGTCCA. Real-time PCR was performed in a total reaction volume of 50 μl containing TaqMan buffer A (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 4mM MgCl₂, 0.25 μM 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 55. Briefly, this PCR Ct value is the cycle number at which emitted fluorescence exceeds 10x the standard deviation (S.D.) of baseline emissions as measured from cycles 3–15. The Ct of the target gene is normalised to the GUS PCR 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: \( \text{mRNA expression} = 2^{(\text{Ct target-Ct GUS})} \times 100\% \).

For RNA interference experiments all targeted and non-targeted siRNA constructs were obtained from Dharmacon (Lafayette, USA) and all experiments were performed in 24 well plates or T25 flasks. THP1/WT, THP1/BTZ (–100) and THP1/BTZ200 cells were cultured following the suppliers’ protocol for THP1 cells. Briefly, prior to transfection, cells were cultured overnight at a density of 5 x 10⁴ cells/ml in RPMI 1640 medium supplemented with 7.5% FCS and 20mM HEPES. Cells were transfected using Dharmafect 2 (DF2) and 100nM of PSMB5 On-Targetplus SmartPool siRNA. As negative control 100nM On-targetplus siControl non-targeting and GAPD pool were used. To assess transfection-efficiency siGLO was used as a transfection-indicator. After 24 hours, transfection-efficiency (usually > 85%) was determined by flow cytometry using siGlo as transfection indicator. At this stage different concentrations of bortezomib were added and induction of apoptosis and cell growth-inhibition was determined after 24 hours bortezomib exposure.
Western blot experiments to assess protein knock-down were performed after 72 hours siRNA incubations.

**Sequence analysis**

DNA was isolated from THP1/WT, THP1/BTZ7, THP1/BTZ30, THP1/BTZ100 and THP1/BTZ(-100) cells using a Qiaamp DNA blood mini kit (250) (Qiagen, Valencia, CA, USA). Subsequently, part the second exon of the PSMB5 gene was amplified by PCR. The primers were designed using Vector NTI (Invitrogen) software (forward : TTCCGCCATGGAGTCATA, reverse : GTTGGCAAGCAGTTTGGGA). PCR products were directly sequenced by dideoxy chain-termination method using a kit ABI Prism™ BigDye Terminator (Perkin Elmer, Foster City, CA, USA) and analyzed by an autosequencer ABI Prism Genetic Analyser 3100 automatic DNA sequencer (Perkin Elmer).

**Growth inhibition assays**

Evaluation of drug sensitivity was carried out as described previously 56. Cells were seeded at an initial density of 1.25 x 10^5 cells/ml in individual wells of a 24-well plate containing up to 50 µl of drug solutions. Inhibition of cell growth was determined after 72 hour incubation at 37°C by counting viable cells based on trypan blue exclusion. The drug concentration required to inhibit cell growth by 50% compared to untreated controls was defined as the IC_{50}.

**Quantification of ubiquitinated proteins/proteasome subunits**

Western blot analysis to determine the accumulation of ubiquitinated proteins upon bortezomib exposure was performed essentially as described previously 56. In short, cells were harvested in the mid-log phase of growth and washed 3 times with ice-cold buffered saline pH 7.4. Total cell lysates of 5 x 10^6 cells were prepared by resuspending in 0.5 ml lysis buffer containing: 50 mM Tris-HCl (pH 7.6), 5 mM dithiotreitol (DTT), 20 µl PIC (Protease Inhibitor Cocktail; 1 tablet/ml H2O), 20% glycerol and 0.5% NP-40. The suspension was sonicated (MSE sonicator, amplitude 7, for 3 x 5 seconds with 20 seconds time intervals at 4°C) and centrifuged in an Eppendorf micro-centrifuge (5 min, 12,000 rpm, 4°C). Protein content of the supernatant was determined by the Bio-Rad protein assay. Twenty to thirty microgram of total cell lysates were fractionated on a 10% polyacrylamide gel containing SDS and transferred onto a PVDF membrane. The membranes were pre-incubated overnight at 4°C in blocking buffer (5% Bio-Rad Blocker in TBS-T; 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20) to prevent non-specific antibody binding. After blocking, the membranes were incubated for 1 hour at room temperature with an ubiquitin-specific antibody (1:1000, Santa-Cruz, SC-8017). After 3 washing steps with TBS-T, the membranes were incubated for 1 hour with HRP-labelled goat-anti-mouse (1:6000, Dako, Glostrup, Denmark) secondary antibody. Detection of...
antibody binding was followed by chemoluminescence using Supersignal (Pierce Biotechnology, Rockford, USA) according to the manufacturers’ instructions. Digital Image acquisition was performed using the Versadoc Imaging System (Biorad Lab., Veenendaal, The Netherlands).

Expression of β1, β2, β5, α7 and αβ-core proteasome subunits were determined essentially as described above. Primary anti-bodies from Biomol for β1 (1:1000, PW8140), β2 (1:1000, PW8145), β5 (1:1000, PW8895 or 1:1000 20S X), α7 (1:1000, PW8110) and αβ (1:1000, PW8155) were incubated for 1 hour at room temperature. An antibody to α-tubulin was used (1:1000, Santa Cruz, sc-8035) to check and normalize for any loading differences. HRP-labelled Donkey-anti-rabbit (1:6000, Amersham, UK) or goat-anti-mouse (1:6000, DAKO) secondary anti-bodies were used. The signal intensity was determined densitometrically using Quantity One software (Bio-Rad) and was expressed relative to the intensity of the α-tubulin signal.

Native gel electrophoresis/gel filtration chromatography
Characterization of the proteasome (detection of core/regulatory particles and suc-Leu-Leu-Val-Tyr-amc hydrolysis activity), in wild type and bortezomib-resistant THP1 cells by native gel electrophoresis was performed essentially as described by Elsasser et al using acid washed glass beads (Sigma) for the preparation of lysates.

Gel filtration chromatography of proteasome complexes and proteasome subunits was performed as described by Chondrogianni et al using a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, The Netherlands) connected to a Gynkotek Model 300 HPLC system and calibrated with a with a mixture of purified proteins in a MW range of 16.6 kD to 669 kD. Briefly, 1.5 x 10^7 THP1/WT and THP1/BTZ100 cells were washed three times in PBS and then lysed in 200 μl ice-cold 20 mM Tris-HCl, pH 7.5, 5 mM ATP and 0.2% Nonidet-P40. Cell extracts were then centrifuged at 4°C for 15 min at 14,000 rpm in an Eppendorf centrifuge. Hundred microliters of the supernatant fraction were applied on the Superdex column equilibrated in 20 mM Tris-HCl, pH7.5, 5 mM ATP and 120 mM NaCl. The column was then eluted with the same buffer at a flow rate of 0.4ml/min. Fractions of 0.4 ml were collected for western blot analysis of proteasome β5 and α7 subunit expression.

Proteasome subunit affinity probing
Affinity labelling of functional proteasome subunits in intact cells was performed with the BodipyFL-Ahx3L3VS probe essentially as described by Berkers et al. Prior to affinity labelling experiments THP1/BTZ cells were cultured in the absence of bortezomib for 4 days.
Mitochondrial trans-membrane potential ($\Delta\psi_m$)

The effect of bortezomib on the mitochondrial trans-membrane potential ($\Delta\psi_m$) was measured as described by Ling et al.\(^3\). Briefly, 1 $\times$ 10\(^5\) cells were resuspended in 0.5 ml culture-medium and incubated for 15 minutes at 37°C with 25 nM tetramethylrhodamine ethyl ester (TMRE), after which cells were washed 3 times with ice-cold PBS supplemented with 0.1% BSA. TMRE accumulation was measured by flow cytometry using a B&D FACScalibur apparatus. Data were analyzed with FCS-express V3 software as described above.

Microarray analysis

THP1/WT cells, THP1/BTZ\(_{30}\), THP1/BTZ\(_{100}\) and THP1/BTZ\(_{(-100, 6mo)}\) cells were harvested in the mid-log phase of growth. The amount and integrity of isolated RNA was measured using a spectrophotometer (NanoDrop ND100 (Wilmington, DE, USA)) and a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Microarray expression analysis was carried out as described by Cloos et al.\(^5\), using the Human Release 2.0 oligonucleotide library (designed by Compugen (San Jose, CA, USA) and obtained from Sigma-Genosys (Zwijndrecht, The Netherlands)) for printing the slides.

The arrays were scanned using an Agilent Microarray Scanner (Agilent Technologies, Amstelveen, The Netherlands). Spot analysis and feature extraction were fully automated using BlueFuse version 3.4 (BlueGnome, Cambridge, UK). Spots were excluded when the Confidence value <0.1. Subsequently, Lowess block normalization was performed within the same program on the non-flagged spots and exported to Excel (Microsoft). Ratio's of resistant divided by the untreated reference sample were used for further analysis.

Immunofluorescence microscopy

Freshly prepared cytospins from THP1/WT and THP1/BTZ\(_{200}\) cells were used at a density of 10,000 cells per spin. Cells were fixed with 3.5% paraformaldehyde for 15 min at RT and then washed twice with PBS and permeabilized by saponin (PBS + 2% saponin, 2 min at RT). Following two successive washes with PBS, slides were incubated for 2 hours at RT with either PBS (control), rabbit anti-β\(_5\) subunit antibody (20S X) diluted 1:50 and mouse anti-α7 subunit antibody (Biomol, PW8110) diluted 1:100 in PBS + 4% BSA. Double staining detection was performed with a FITC-conjugated goat anti-mouse antibody (Sanquin, Amsterdam, The Netherlands) diluted 1:100 and TRITC-conjugated swine anti-rabbit antibody (Dako, Glostrup, Denmark) diluted 1:100. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined using a Leica fluorescence microscope.
**Apoptosis assay**

Bortezomib-induced apoptosis was analyzed by measuring AnnexinV-FITC/7-amino-actinomycin D (7-AAD) staining (APOPTEST™-FITC A700, VPS Diagnostics, Hoeven, the Netherlands) according to the instructions of the manufacturer. Briefly, induction of apoptosis was determined after 24 hours exposure by bortezomib. One million cells were harvested and washed 3 times with ice-cold PBS. The cell pellet was incubated for 30 minutes with 7-Amino-actinomycin D (7-AAD) on ice followed by incubation with Annexin-V according to the instructions of the manufacturer. Annexin-V and 7-AAD expression was measured by flow cytometry (Beckton & Dickinson, FACScalibur) and analysed using FCSexpress V3 software (Denovo software, Thornhill, Canada).

**Statistics**

Statistical analysis was performed using Analysis of Variance between groups (ANOVA) in Graphpad prism. *P* values < 0.05 were considered to be statistically significant.
**Supplementary Figures**

**Supplementary Figure 1.** Western blot analysis of MRP1-6, Pgp and BCRP in THP1/WT, THP/BTZ30 and THP1/BTZ200 cells. As positive controls served drug-selected or transfected cell lines as described by Schef-fer et al 43. A representative picture of 2 separate experiments is depicted.
Supplementary Figure 2. Ratios of gene expression levels in THP1/BTZ30, THP1/BTZ100 and THP1/BTZ(-100) cells relative to THP1/WT cells are depicted for selected genes implicated in drug metabolism, resistance, apoptosis and cell cycle regulation, and compared with those in the ubiquitin proteasome pathway. Experimental details are described in the Materials & Methods section.
### Bortezomib resistance & mutant PSMB5 up-regulation

#### Gene expression

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#### Genes implicated in drug resistance and metabolism (101 genes)

- ATP-Binding Cassette transporters (ABCA1-A9, A12, B1, B4, B7-B10, C1-C13)
- ATP-Binding Cassette transporters (ABCD1-D4, E1, F1, F2, G1, G2, G4, G5, G6)
- B-cell CLL/lymphoma 2 (BCL2)
- BCL2-like 1 (BCL2L1)
- Bleomycin hydrolase (BLMH)
- Breast cancer 1, early onset (BCRA1)
- Breast cancer 2, early onset (BCRA2)
- Catechol-O-methyltransferase (COMT)
- Cellular retinoic acid binding protein 1 (CRABP1)
- Cytochrome P450, family 1, subfamilies (CYP1A1,1A2,2B6,2C9,2D6,2E1,3A5)
- Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4)
- Dihydrololate reductase (DHFR)
- Epidermal growth factor receptor (EGFR)
- ELK1, member of ETS oncogene family (ELK1)
- Epoxide hydrolase 1, microsomal (xenobiotic) (EPHX1)
- Epoxide hydrolase 2, cytoplasmic (EPHX2)
- V-erb-b2 erythroblast leukemia viral oncogene homologs (ERBB2,3)
- Excision repair cross-complementing rodent repair deficiency (ERCC3)
- Estrogen receptors (ESR1,2)
- Fibrinogen gamma (FGF2)
- V-fos FBJ murine osteosarcoma viral oncogene homolog (FOS)
- Glycogen synthase kinase 3 alpha (GSK3A)
- Glutathione S-transferase pi (GSTP1)
- Hypoxia-inducible factor 1, alpha subunit (HIF1A)
- Insulin-like growth factor receptors (IGF1R,2R)
- Met proto-oncogene (hepatocyte growth factor receptor) (MET)
- O-6-methylguanine-DNA methyltransferase (MGMT)
- MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli) (MLH1)
- MutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli) (MSH2)
- MutS homolog 3 (E. coli) (MSH3)
- Major vault protein (MVP)
- N-acetyltransferase 2 (arylamine N-acetyltransferase) (NAT2)
- NAD(P)H dehydrogenase, quinone 1 (NQO1)
- Peroxisome proliferator-activated receptor (PARRdelta,gamma)
- Peptidylprolyl isomerase A (cyclophilin A) (PPIA)
- Retinoic acid receptor (RARalpha, beta, gamma)
- Retinoblastoma 1 (including osteosarcoma) (RB1)
- Nuclear factor of kappa B (REL)
- Ribosomal protein L13aa (RPL13A)
- Retinoid X receptor, alpha (RXRA,B,G)
- Superoxide dismutase 1 (SOD1)
- Tumor necrosis factor receptor superfamily, member 11a (TNFRSF11A)
- Topoisomerase (DNA) I (TOP1,2A,2B)
- Tumor protein p53 (Li-Fraumeni syndrome) (TP53)
- Thymidine S-methyltransferase (TPMT)
- Xeroderma pigmentosum, complementation group A and C (XPA,C)

Supplementary Figure 2.
Chapter 4

<table>
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<th>gene expression</th>
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**Apoptosis (59 genes)**

- BCL2-antagonist of cell death (BAD)
- BCL2-antagonist/killer 1 (BAK)
- BCL2-associated X protein (BAX)
- B-cell CLL/lymphoma 2 (BCL2)
- BCL2-like 1 (BCL2L1)
- BCL2-like 11 (apoptosis facilitator) (BCL2L11)
- BH3 interacting domain death agonist (BID)
- Baculoviral IAP repeat-containing (BIIRC1-4)
- Baculoviral IAP repeat-containing 5 (survivin) (BIIRC5)
- BCL2/adenovirus E1B 19kDa interacting protein 3-like (BNIP3L)
- Caspases (CASP1-10)
- Conserved helix-loop-helix ubiquitous kinase (CHUK)
- Cytochrome c, somatic (CYCS)
- DNA fragmentation factor, 45kDa, alpha polypeptide (DFFA)
- DNA fragmentation factor, (caspase-activated DNase) (DFFB)
- Fas (TNFRSF6)-associated via death domain (FADD)
- Fas ligand (TNF superfamily, member 6) (FAS)
- Helicase, lymphoid-specific (HELLS)
- Harakiri, BCL2 interacting protein (contains only BH3 domain) (HRK)
- Inhibitor of kappa B kinase (IKKBbeta,gamma)
- Interferon regulatory factors (IRF1-7)
- Jun oncogene (JUN)
- Lymphotoxin alpha (TNF superfamily, member 1) (LTA)
- Mitogen-activated protein kinase kinase 4 (MAP2K4)
- Mitogen-activated protein kinase kinase kinase 1 (MAPK3K1)
- Mitogen-activated protein kinase kinase kinase 4 (MAPK3K1)
- Mdm2, double minute 2, p53 binding protein (mouse) (MDM2)
- Nuclear factor of kappa B (p105) (NFKB1)
- Nuclear factor of kappa B inhibitor alpha (NFKBIA)
- Nuclear factor of kappa B inhibitor beta (NFKBIB)
- Nuclear factor of kappa B inhibitor epsilon (NFKBIE)
- Perforin 1 (pore forming protein) (PRF1)
- Nuclear factor of kappa B, p65 (RELA)
- Receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIPK1)
- Tumor necrosis factor (TNF superfamily, member 2) (TNF)
- Tumor necrosis factor receptor superfamily, member 10b (TNFRSF10b,1A,21)
- Tumor necrosis factor receptor superfamily, member 1B (TNFRSF1B)
- Pleckstrin homology domain containing, family G member 5 (PLEKNG5)
- Tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10)
- Tumor protein p53 (Li-Fraumeni syndrome) (TP53)
- Tumor protein p73 (TP73)
- TNF receptor-associated factors (TRAF1-3)

Supplementary Figure 2.
## Supplementary Figure 2.

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<th>gene expression</th>
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### Cell Cycle (76 genes)

- ABL1
- ATM
- BUB1
- CDC20
- CDC25A
- CDC25C
- CDC45
- CDC6
- CDC7
- CDH1
- CDKN1A
- CDKN2A,D
- CHEK1
- CHEK2
- DTX4
- E2F1
- E2F2
- E2F3
- E2F4
- E2F5
- ESPL1
- GADD45A
- GSK3B
- HDAC1-8
- MAD1
- MCM2,3,5,7
- MCM6
- MDM2
- ORC1L,2L,3L,4L,5L,6L
- PCNA
- PLK1
- PRKDC
- PTPRA
- PRGG1
- PTTG2
- RBL1
- SKP2
- SMAD4
- TFDP1
- TP53
- WEE1

Supplementary Figure 2.
**Supplementary Figure 3.** Western blot analysis of hsp27, hsp90, apoptosis inhibitor protein XIAP and p21 (cyclin dependent kinase inhibitor 1A) in THP/WT cells and bortezomib-resistant THP1 cells. Note: induction of p21 in THP1/BTZ500 cells point to early growth inhibitory effects of 500 nM bortezomib in these cells. A representative picture of 2 separate experiments is shown.

**Supplementary Figure 4.** Fluorescence microscopy of: (A)/(D); a7 proteasome subunits, (B)/E); b5 proteasome subunits and (C)/(F); merge of the a7 and b5 staining. Upper panels: THP1/WT cells; lower panels: THP1/BTZ200 cells.
Supplementary Figure 5. Human CCRF-CEM cells of T cells origin were chronically exposed to stepwise increasing concentrations of bortezomib over a period of 6 months. Expression levels of proteasome subunit β5 and 7 are depicted for THP1/WT cells and two selected bortezomib resistant variants grown at 7 nM bortezomib (BTZ7) and 200 nM bortezomib (BTZ200).
Chapter 5

Impaired bortezomib binding to mutant β5 subunit of the proteasome is the underlying basis for bortezomib resistance in leukemia cells

Niels E. Franke, Denise Niewerth, Yehuda G. Assaraf, Johan van Meerloo, Katarina Vojtekova, Christina H. van Zantwijk, Sonja Zweegman, Elena T. Chan, Christopher J. Kirk, Daan P. Geerke, Aaron D. Schimmer, Gertjan J.L. Kaspers, Gerrit Jansen and Jacqueline Cloos

Leukemia. 2012 Apr;26(4):757-68.
ABSTRACT

Proteasome inhibition is a novel treatment for several hematological malignancies. However, resistance to the proteasome inhibitor bortezomib (BTZ, Velcade®) is an emerging clinical impediment. Mutations in the β5-subunit of the proteasome, the primary target of BTZ, have been associated with drug resistance. However, the exact mechanism by which these mutations contribute to bortezomib resistance, is still largely unknown. Towards this end, we here developed BTZ-resistant multiple myeloma (8226) and acute lymphoblastic leukemia (CCRF-CEM) cell line models by exposure to stepwise increasing concentrations of BTZ. Characterization of the various BTZ-resistant cells revealed up-regulation of mutant β5-subunit of the proteasome. These newly identified β5-subunit mutations, along with previously described mutations, formed a mutation cluster region in the BTZ-binding pocket of the β5-subunit, that of the S1 specificity pocket in particular. Moreover, we provide the first evidence that the mechanism underlying BTZ-resistance in these tumor cells is impaired binding of BTZ to the mutant β5-subunit of the proteasome. We propose that proteasome subunit overexpression is an essential compensatory mechanism for the impaired catalytic activity of these mutant proteasomes. Our findings further suggest that second generation proteasome inhibitors that target the α7 subunit of the proteasome can overcome this drug resistance modality.
INTRODUCTION

The ubiquitin proteasome system (UPS) controls multiple key cellular homeostasis processes including cell cycle, signal transduction, DNA repair, immune and inflammatory responses, growth and differentiation. Proteins targeted for degradation are tagged with multiple ubiquitin residues by a variety of E1, E2 and E3 ligases and degraded by the 26S proteasome. This latter complex consists of a 19S lid complex and a 20S core complex. The 19S is responsible for recognizing and unfolding ubiquinated proteins, whereas the 20S complex carries out the subsequent cleavage of the unfolded protein. The barrel-shaped 20S complex consists of two alpha and two beta subunit rings, each of which contains 7 different α or β subunits. Upon immune stimulation by e.g. interferon-γ, the constitutive catalytic subunits β1 (PSMB6), β2 (PSMB7) and β5 (PSMB5), are replaced by their immune cognate forms; β1i (PSMB9), β2i (PSMB10) and β5i (PSMB8).5,6 Recently it has been shown that these immune forms are highly efficient in protein degradation, thus preventing aggresome formation.8

The boronic dipeptide bortezomib (BTZ, Velcade™) inhibits the UPS by reversibly inhibiting the chymotrypsin-like activity of the β5-subunit of the proteasome and to a lesser extent also the caspase-like activity of the β6-subunit9,10. As a result of proteasome inhibition, BTZ induces cell death through various mechanisms including inhibition of NFκB activity11, activation of p5312, accumulation of misfolded proteins13, c-Jun N-terminal kinase (JNK) activation14 and stabilization of cell cycle inhibitors.15 BTZ has shown promising clinical responses in multiple hematological malignancies including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), lymphoma and in particular multiple myeloma (MM), where BTZ is currently used as the first line chemotherapeutic treatment (reviewed in16-18). Despite the good initial clinical response, some of the MM patients eventually become refractory or relapse after BTZ treatment.17,19 In addition, BTZ resistance also seems to occur in ALL.20 Different mechanisms of BTZ resistance have been suggested; Kraus et al.21 hypothesized, based on a BTZ-resistant AML cell line model and ex vivo primary patient samples, that quantitative alterations in the ratio between β2-type and (β1 + β5)-type of active proteasomal polypeptides induce BTZ resistance. Rückruch et al.22 showed upregulation of the proteasomal machinery in BTZ-resistant AML and MM model cell lines. In addition to upregulation, we as well as others have identified single point mutations in PSMB5, which encodes for the β5 subunit of the proteasome, the primary target of BTZ, in BTZ-resistant AML, ALL and MM cell lines.1,4,7

Here we provide the first evidence that the mechanism underlying BTZ-resistance in ALL and MM cells is impaired binding of BTZ to a mutant β5-subunit of the proteasome. This is further substantiated by the identification of multiple novel β5-subunit mutations in various BTZ-resistant cell lines, which form a mutation cluster region in the
BTZ-binding pocket of the β5-subunit of the proteasome or its close vicinity. We propose that proteasome subunit overexpression is a compensatory mechanism for the impaired catalytic activity of such mutant proteasomes.

MATERIALS AND METHODS

Cell culture and development of BTZ-resistant cell lines

Human T-ALL CCRF-CEM cells and human multiple myeloma RPMI-8226 cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium containing glutamine (Invitrogen/Gibco (Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), and 100 μg/ml penicillin/streptomycin (Invitrogen) at 5% CO2 and 37°C. Cell cultures were seeded at a density of 3 x 10^5 cells/ml and refreshed twice weekly. Bortezomib (BTZ)-resistant CCRF-CEM and RPMI-8226 cells were obtained by stepwise increasing extracellular concentrations of BTZ over a period of 4 to 15 months, respectively.

Computational modeling of β5 mutations

For the mutation modeling analysis, the yeast proteasomal crystal structure in complex with BTZ (MMDB ID: 38209, PDB ID: 2F16)\textsuperscript{23}, the yeast proteasomal crystal structure in complex with epoxomicin (MMDB ID: 14501, PDB ID: 1G65\textsuperscript{24} and the bovine proteasomal crystal structure (MMDB ID: 19465, PDB ID: 1IRU)\textsuperscript{25} were used as templates. BTZ was manually docked into the bovine β5 subunit using Molecular Operating Environment (MOE) v2009 (Chemical Computing Group, Montreal, Quebec, Canada) and energy-minimized using the Amber99 force-field. The β5 and β6 subunit together with crystal water molecules within a radius of 4.5 Å from any atom of the ligand were used for further analysis. Hydrogen atoms were added using the corresponding tool in MOE. The peptide backbone of epoxomicin was used as scaffold for the generation of the coordinates of the substrate suc-LLVY-AMC. Missing side chains of this substrate were added using the builder tool of MOE and after addition of hydrogen atoms, its structure was energy minimized in complex with the β5 and β6 subunit of the yeast proteasome. Subsequently, the suc-LLVY-AMC was manually docked into the β5 subunit binding pocket within the bovine crystal structure. Mutations were manually introduced in the thus obtained BTZ-bound and suc-LLVY-AMC-bound complexes, followed by an exploration of the possible orientations of the newly introduced amino-acid side chains (using the rotamer-explorer utility of MOE) and subsequent energy minimization. All energy minimizations were performed using the Amber99 force field as implemented in MOE.
Supplemental data

Information on antibodies and drugs, cell growth inhibition, immunoblotting (Western blotting and native gels), RT-PCR and sequence analysis can be found in Document S1 and Table S1.

RESULTS

Generation of BTZ-resistant cell lines

In order to obtain BTZ-resistant model cell lines, human T-ALL CCRF-CEM and the MM 8226 cell lines were exposed to gradually increasing concentrations of BTZ starting at 0.2nM of BTZ. Consequently, the BTZ-resistant sublines of CCRF-CEM and 8226 stably growing in the presence of 7 nM (CEM/BTZ7 and 8226/BTZ7), 100 nM (8226/BTZ100), and 200 nM BTZ (CEM/BTZ200) were used for characterization of the underlying mechanism of BTZ-resistance. Figure 1a summarizes the distinct time-course of acquisition of BTZ-resistance in the various drug selected cell lines. CCRF-CEM cells rapidly acquired drug-resistance to a concentration of 200 nM of BTZ within 4 months. Similarly, the previously published human AML THP-1 cell line required 6 months to achieve a drug resistance level of 200 nM BTZ.1 In contrast, acquisition of BTZ-resistance in the MM cell line 8226 was much slower as cells required 15 months to become resistant to 100 nM BTZ. Cytotoxicity assays revealed 10-fold and 170-fold BTZ resistance in CEM/BTZ7 and CEM/BTZ200 cells, respectively, compared with parental CEM/WT cells (IC50: 1.5 ± 0.4 nM; Figure 1b). 8226/BTZ7 and 8226/BTZ100 displayed 5-fold and 43-fold BTZ resistance (IC50: 2.6 ± 0.3 nM; Figure 1c). Together, these studies might imply that acute leukemia cells were more prone to become BTZ-resistant than MM cells.

Cross-resistance profile of bortezomib-resistant cells to proteasome inhibitors

We explored the sensitivity of BTZ-resistant cell lines to an assortment of proteasome inhibitors. Table 1 depicts the IC50 values of BTZ-resistant sublines of which the BTZ-resistant CCRF-CEM cells exhibited a markedly increased resistance towards a panel of proteasome inhibitors; the most striking cross-resistance was observed towards MG132 (CEM/BTZ200 vs CEM/WT; 122 fold) and an epoxyketone-based irreversible proteasome inhibitor that is orally bioactive; ONX 091226 (CEM/BTZ200 vs CEM/WT cells; 147 fold). Moreover, CEM/BTZ200 cells gained 39-fold and 25-fold resistance against two other second generation irreversible proteasome inhibitors carfilzomib27 and the immunoproteasome inhibitor ONX 091428, respectively. This resistance level was lower than that observed for the parent drug BTZ (170 fold). CEM/BTZ7 and CEM/BTZ200 cells retained full sensitivity towards the proteasome inhibitor 5AHQ which binds to the α7 subunit of the proteasome.29 Similar results were observed in the MM BTZ-resistant 8226 sublines. 8226/BTZ100 cells
Figure 1. Generation of BTZ resistant cell line models. (A) Timeline of the generation of BTZ resistant human monocytic/macrophage THP1 cells, human T-acute lymphoblastic leukemia CEM cells and human multiple myeloma 8226 cells. The timeline depicts dose-increments of BTZ during the acquisition of resistance. (B) Dose-response curve for bortezomib-induced growth inhibition of wild-type (WT) CEM and bortezomib (BTZ)–resistant variants CEM/BTZ7 and CEM/BTZ200 and (C) 8226/WT, 8226/BTZ7 and 8226/BTZ100. BTZ-resistant cells were selected for growth in extracellular concentrations of 7 nM, 100 nM, and 200 nM bortezomib, respectively. Results depicted are the mean of 3-6 separate experiments (±SEM), measured after 96 hours drug exposure by MTT cytotoxicity assay. Abbreviation: LCG = Leukemic cell growth

Table 1. Growth inhibitory effects of various proteasome inhibitors for wild-type (WT) and bortezomib (BTZ)–resistant cells

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<td>BTZ (nM)</td>
<td>2.6 ± 0.3</td>
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<td>MG132 (nM)</td>
<td>307.8 ± 165</td>
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<td>MG262 (nM)</td>
<td>6.7 ± 2.3</td>
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<td>4A6 (nM)</td>
<td>133.2 ± 115.6</td>
<td>1412 ± 283 (8.7)</td>
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<td>CFZ (nM)</td>
<td>2.4 ± 0.8</td>
<td>2.6 ± 0.7 (1.2)</td>
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<td>ONX 0912 (nM)</td>
<td>122 ± 39</td>
<td>160 ± 20 (1.5)</td>
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<td>ONX 0914 (nM)</td>
<td>26.0 ± 7.5</td>
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<td>SAHQ (μM)</td>
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showed a marked cross-resistance towards all tested proteasome inhibitors that target the β-subunits, in particular to ONX 0914 (60-fold). Interestingly, 8226/BTZ7 cells largely retained parental cell sensitivity towards the second generation proteasome inhibitor carfilzomib, the orally bioactive proteasome inhibitor ONX 0912, as well as the immunoproteasome inhibitor, ONX 0914. Consistent with BTZ-resistant CCRF-CEM cells, both 8226/BTZ7 and 8226/BTZ100 retained parental cell sensitivity to the α7 subunit-targeted proteasome inhibitor, 5AHQ. Hence, both CEM/BTZ200 and 8226/BTZ100 displayed cross-resistance to proteasome inhibitors. Differences in cross-resistance levels between CEM/BTZ200 and 8226/BTZ100 were most prominently found for MG132, carfilzomib and ONX 0912 (see Table 1). Together, these results demonstrate that cross-resistance of both ALL and MM bortezomib-resistant cells is restricted to drugs that target the β-subunits of the proteasome, while retaining sensitivity to proteasome inhibitors (5AHQ) that bind to β-subunit-independent components of the proteasome. Dissimilarities in cross-resistance between the CEM/BTZ100 and 8226/BTZ100 to the various proteasome inhibitors may indicate distinct β-subunit related resistance mechanisms.

**Upregulation of constitutive beta subunits of the proteasome in bortezomib-resistant cells**

BTZ displays a low nM inhibitory constant of the catalytic chymotrypsin-like activity of the eukaryotic proteasome. Consistently, BTZ targets the β5-subunit of the proteasome at the low nM concentration range in these cell lines, whereas at higher concentrations, it also inhibits the β1 subunit. Since our cross-resistance data (Table 1) suggested a β-subunit-specific resistance mechanism, the expression pattern of the β1, β2 and β5-subunits of the proteasome was determined at the RNA and protein levels. Figure 2a depicts the mRNA expression levels of PSMB6 (β1), PSMB7 (β2) and PSMB5 (β5) as normalized to GUS; only a minor elevation of all constitutive catalytic subunits was observed in both CEM/BTZ7 and CEM/BTZ200 cells. A more profound upregulation of the mRNA for constitutive catalytic subunits was seen in BTZ-resistant MM 8226 cells. Moreover, 8226/BTZ100 cells showed a selective 15-fold upregulation of the PSMB5 gene. Subsequently, the expression profile of the proteasome catalytic subunits was determined at the protein level (Figure 2b). BTZ-resistant CEM cells displayed an upregulation of the constitutive catalytic β-subunits, concurrent with elevated mRNA levels. In addition, an upregulation of the non-catalytic α7 subunit was also observed, indicating an upregulation of the whole 20S proteasome. In contrast to parental 8226 cells, BTZ100-resistant 8226 cells showed a more selective upregulation of the β5 subunit, while only a minor increment in the expression of the other subunits was observed. Intriguingly, this high mRNA level of the β5-subunit was not substantiated with higher proteins being comparable to the level of BTZ7. This may imply that additional post-transcriptional mechanisms contribute to the resistance.
Figure 2. Upregulation of proteasomal β subunits expression in BTZ resistant cells. (A) mRNA expression levels of β1, β2 and β5 subunit in BTZ resistant CEM/BTZ7, CEM/BTZ200, 8226/BTZ7 and 8226/BTZ100 relative to their corresponding WT cells. Results depicted are the mean of 3-5 separate experiments (±SEM). (B) Protein expression levels of proteasome β1, β2, β5 and α7 subunits in WT and BTZ resistant lines as determined by conventional western blot. Cells were cultured without BTZ (-) or with 7 to 200 nM of BTZ for 24 hours. α-tubulin was used as loading control. (C/D) Native gel electrophoresis of crude cell extracts (equal protein loading) of WT and BTZ resistant lines subsequently analyzed for β5 and α7 protein expression (C) or β5i and α7 (D). Extracts used for Figure D were obtained from cells incubated without BTZ (-) or with 7 to 200 nM of BTZ for 24 hours. The upper band in the Figures C and D represent the 20S proteasome core particle (CP) in complex with the regulatory 19S particle (RP), the lower band represents the CP alone. (E) Relative expression of β5 and β5i protein in WT and BTZ resistant lines as determined by ELISA.
Mutations in β5 subunit impair bortezomib binding

To confirm that the change in subunit expression also results in a parallel change in proteasome complex composition, native gel electrophoresis was performed as previously described by Elsasser et al.30 Western blot analysis performed after native gel electrophoresis is shown in Figure 2c. Concurrent with the denaturing gel electrophoresis data (Figure 2b), native gel electrophoresis also detected upregulation of both the β5 and α7 subunits in CEM/BTZ cells, indicating an upregulation of the entire 20S complex. Moreover, 8226/BTZ cells also showed a specific upregulation of the β5- subunit of the proteasome, which is incorporated in the 20S complex. The 20S proteasome consists of two α and two β-rings. Since one β ring consists of seven different β-subunits, only one β5 subunit can be incorporated per β-ring.25 Therefore, a shift in expression might occur from immunoproteasome towards constitutive proteasome expression. Figure 2e shows the relative expression of constitutive as well as the immunoproteasome β5 subunit (β5i) using an ELISA approach as described previously.31 In BTZ-resistant 8226 cells, a remarkable shift in expression of the β5i to constitutive β5 subunit was observed. In contrast, BTZ-resistant CEM cells showed no alteration in the ratio between immune and constitutive proteasome subunit; these results were confirmed using native gel electrophoresis (Figure 2d). Hence, these findings reveal decreased incorporation of immune β5i subunit in the BTZ-resistant 8226 cells, which exhibited a near complete substitution of the immunoproteasome by the constitutive proteasome. Alternatively, drug-resistant CEM cells show a similar upregulation of both β5i and β5.

Identification of multiple mutations in the PSMB5 gene in 8226/BTZ and CEM/BTZ cells

We sequenced the PSMB5, PSMB6 and PSMB7 (encoding for the β5, β1 and β2 subunits, respectively) at the genomic DNA level. The low BTZ-resistant CEM/BTZ7 cells harbored a mutation in the PSMB5 gene; a single nucleotide substitution (G332T) was identified hence resulting in a cysteine to phenylalanine substitution at amino acid position 52 of the β5 subunit. Moreover, CEM/BTZ200 cells gained an additional mutation (C323T), resulting in an Ala49Val substitution in the β5 subunit. The latter mutation is a substitution at the same position previously published in THP1/BTZ100 cells, only resulting in a different amino acid substitution (Ala49Thr). This amino acid has been described by Groll et al.23 to be directly involved in BTZ binding. The position of the first mutation (Cys52Phe) has not been described as being involved in direct BTZ binding. However, it resides in a close proximity to Met45; it has been suggested that this latter amino acid undergoes a conformational change prior to BTZ binding to the β5 subunit.23

Initially, the low level drug-resistant 8226/BTZ7 cells had no detectable mutation in the PSMB5 gene. However, upon prolonged culturing of these BTZ-resistant cells in the presence of 7nM BTZ for several weeks, a mutation (A247G) emerged that resulted in a Thr21Ala substitution in the β5 subunit. 8226/BTZ100 cells lost this Thr21Ala mutation,
but gained the Ala49Thr substitution that was originally identified in THP1/BTZ100 cells. Both Thr21 and Ala49 reside in the BTZ-binding pocket of the β5 subunit of the proteasome and were previously identified as being directly involved in BTZ binding.23 Sequencing of the PSMB6 and PSMB7 genes did not reveal any mutations in both parental CEM and 8226 cells, and BTZ-resistant counterparts. Likewise, no PSMB5 gene mutations were identified in parental cells grown in parallel with 8226 and CEM cells during the course of exposure to increasing BTZ concentrations (results not shown).

To distinguish between the expansion of a pre-existing β5 subunit mutant subclone and the de novo acquisition of a mutation and its subsequent outgrowth, we generated a second and independent panel of BTZ-resistant CEM and THP1 cells termed CEM/BTZ100N and THP1/BTZ100N, respectively. These new BTZ-resistant cell lines were established using precisely the same drug selection protocol employed for the original BTZ-resistant cell lines. Interestingly, different mutations were found in the newly obtained cell lines; THP1/BTZ100N cells acquired an A310G mutation resulting in a Met45Val substitution in the β5 subunit. Remarkably, the THP1/BTZ500 cell line had the same Met45Ile substitution. Furthermore, CEM/BTZ100N cells displayed the same G322A mutation as observed in 8226/BTZ100 and THP1/100, but this mutation was not previously seen in CEM/BTZ200 cells. A summary of the multiple β5-subunit mutations is depicted in Table 2. Hence, these data indicate that prolonged BTZ exposure results in de novo acquisition of multiple mutations in the PSMB5 gene, thereby resulting in amino acid substitutions within the BTZ binding pocket, that are presumably directly or indirectly involved in BTZ binding.

### Table 2. Overview of mutations in PSMB5 gene in BTZ-resistant cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BTZ-resistant selectant</th>
<th>Location mutation in PSMB5&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino acid substitution&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEM</td>
<td>BTZ7</td>
<td>G332T</td>
<td>Cys52Phe</td>
</tr>
<tr>
<td></td>
<td>BTZ200</td>
<td>G332T &amp; C323T</td>
<td>Ala49Val &amp; Cys52Phe</td>
</tr>
<tr>
<td></td>
<td>BTZ30-100N</td>
<td>G322A</td>
<td>Ala49Thr</td>
</tr>
<tr>
<td>8226</td>
<td>BTZ7</td>
<td>A247G</td>
<td>Thr21Ala</td>
</tr>
<tr>
<td></td>
<td>BTZ100</td>
<td>G322A</td>
<td>Ala49Thr</td>
</tr>
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<td>THP-1</td>
<td>BTZ100</td>
<td>G322A</td>
<td>Ala49Thr</td>
</tr>
<tr>
<td></td>
<td>BTZ500</td>
<td>G322A &amp; G311T</td>
<td>Ala49Thr &amp; Met45Ile</td>
</tr>
<tr>
<td></td>
<td>BTZ7-100N</td>
<td>A310G</td>
<td>Met45Val</td>
</tr>
</tbody>
</table>

<sup>a</sup>NM_002797 was used as reference sequence.

<sup>b</sup>Amino acid positions were calculated using the NP_002788 sequence, at the start of the processed proteins (starting at Thr60 of the unprocessed protein)

Mutations identified in the current study as well as in previous studies formed a mutation cluster region at both the nucleotide and the protein levels (Figure 3a and 3b). Although the Thr21Ala mutation seemed to be an outlier, when mutations are positioned within the 3D-structure of the proteasome’s β5 subunit, all mutations cluster around
the BTZ binding pocket (Figure 3c). Moreover, the vast majority of the mutations reside around the S1 specificity pocket of the β5 subunit. This S1 specificity pocket is mainly responsible for recognizing the peptide bond of the substrate that is to be cleaved.\textsuperscript{23,32} The first side chain (P1) of proteasome’s substrates as counted from the Thr1 binding residue, binds to the S1 specificity pocket. Part of the specificity of proteasome inhibitors is therefore mediated by the P1 side chain of the drug.\textsuperscript{33} As a result, mutations in the S1 pocket can have crucial adverse consequences for proteasome inhibitor binding.

**Figure 3. Mutations in the β5 subunit form a cluster region.** Mutations described in the current study cluster together with previously published papers in DNA (A) and protein sequence (B). Represented mutations are described in: ♦ current paper, ∇ Oerlemans and Franke et al.,\textsuperscript{1} Δ Lu et al.,\textsuperscript{3} ○ Lu et al.,\textsuperscript{4} ∠ Ri et al.\textsuperscript{7} (C) Graphic representation of the 3D protein-backbone structure of the β5 subunit. Mutations are depicted in green. P1 = Substrate side chain 1, S1 = specificity binding pocket 1.

**Acquired β5 subunit mutations induce amino acid substitution in the BTZ-binding pocket that hinder BTZ binding**

To investigate the influence of mutations on the BTZ binding capacity, we performed an \textit{in silico} 3-D modeling analysis of the mutated β5 subunit. To this end, we computationally explored the effect of the mutations on BTZ binding, using the crystal structure of the yeast 20s proteasome in complex with BTZ\textsuperscript{23}, and using the complex structure obtained by manually docking BTZ into the wild type bovine proteasome crystal structure\textsuperscript{25}. Subsequently, mutations were manually introduced as described in Materials and Methods. Figure 4a shows the predicted position of BTZ binding to the wild type bovine β5 subunit, as well as critical residues involved in this binding.
In CEM/BTZ7 cells we identified a Cys52Phe substitution; as previously described by Groll et al., Cys52 is not known to be directly involved in BTZ binding. However, it resides in the S1 binding pocket of β5 subunit. Our analysis shows that introduction of a Phe group at position 52 would lead to a slight repulsion of BTZ from the S1 pocket (Figure 4b). Furthermore, CEM/BTZ200 acquired an additional Ala49Val substitution. The amino acid at this position has been found to be directly involved in BTZ binding and has been described by Lu et al. to be involved in conferring BTZ-resistance. After introducing the Ala49Val mutation into the Cys52Phe mutant, we did not observe a direct steric clash of the additional methyl groups and BTZ atoms, although the distance between the methyl group and the BTZ P1 side chain, is only 3.3Å. Moreover, Heinemeyer et al. suggested that Ala49Val substitution restricts accessibility to the S1 pocket. Therefore, introducing residues with a larger side chain might negatively affect BTZ binding, by hampering the accessibility of P1 to the S1 pocket. This is illustrated by our structural analysis of ligand binding to the Ala49Thr mutant (see below).

8226/BTZ7 cells harbored a previously undescribed Thr21Ala mutation. This mutation was initially heterozygous, but after prolonged exposure to 7nM BTZ, it became homozygous. Thr21 has been described by Groll et al. to be directly involved in BTZ binding. Substitution of this residue will result in a loss of a protein-ligand hydrogen bond (compare Figures 4a and 4c), which is likely to decrease BTZ affinity to the β5 subunit. The 8226/BTZ7 cell line was still heterozygous when BTZ concentrations were increased towards 100nM, whereas upon the establishment of 8226/BTZ100 cells, the Thr21Ala substitution was lost and replaced by the Ala49Thr substitution. This latter mutation has been described previously in the BTZ-resistant Jurkat (T-ALL), THP-1 (AML) cell lines and recently also in a BTZ-resistant 8226 MM cell line. This amino acid has been also described to be directly involved in BTZ-binding via a hydrogen bond network between Ala49, Ala50, Asp114 of the β6 subunit and a well defined H2O molecule. Depending on the orientation of its side chain, either steric interference with the BTZ backbone (Figure 4d) or closure of the S1 pocket (Figure 4e) was observed upon introducing Thr49. Both effects are expected to have a negative influence on BTZ binding to the β5 subunit.

Mutations on the Met45 position did not reveal a direct effect on BTZ binding in our in silico analysis. However, this amino acid has been previously described to be crucial in determining S1 pocket specificity. Moreover, this amino acid has to undergo a conformational change upon BTZ binding, thereby resulting in an induced fit. Substitutions at this Met45 can therefore have a marked impact on the dynamics of BTZ binding by altering the binding pocket’s specificity and flexibility. Together, the in silico data suggest that all acquired mutations decrease the affinity of the β5 binding pocket to BTZ, and particularly of the S1 binding pocket.
Mutations in β5 subunit impair bortezomib binding

Figure 4. In silico 3D-modeling analysis of mutations in β5 subunit shows decreased BTZ binding capacity. Prediction of the binding pose of BTZ (dark-purple) in complex with (A) WT Bos Taurus β5 subunit compared to (B) Cys52Phe, (C) Thr21Ala, and (D/E) Ala49Thr mutated β5 subunit. The latter two figures represent two different conformations after rotamere analysis of the Ala49 amino acid side chain. Critical residues involved in binding are depicted in green. Heavy atoms involved in hydrogen bonds are connected by dotted grey lines.
Mutations in β5 subunit hinder suc-LLVY-AMC binding

Since the S1 pocket of the β5 subunit is crucial for substrate recognition, we hypothesized that acquired mutations in BTZ-resistant cells are likely to affect substrate binding that is widely used in studies on proteasome’s catalytic activity. We performed in silico 3D modeling analysis of the β5 subunit tetrapeptide N-Succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (suc-LLVY-AMC). For our in silico 3D structural studies, we employed a crystal structure of the yeast proteasome in complex with epoxomicin and we used epoxomicin as a scaffold to generate atomic coordinates for bound suc-LLVY-AMC substrate. In addition, the substrate was docked into the wild type bovine crystal structure of the proteasome, after which we introduced the different β5 subunit mutations. Figure 5a shows the prediction of the binding pose of the suc-LLVY-AMC substrate in the WT bovine β5 subunit. The P1 Tyr residue of the substrate binds to the S1 specificity pocket of the β5 subunit. Introducing the Cys52Phe mutation into the complex shows that the P1 Tyr group of the substrate is displaced by Phe52 (Figure 5b), which will negatively affect binding affinity. Moreover, the conformational change of Met 45 necessary for substrate binding (as discussed above), might also be hindered. The additional introduction of Ala49Val was found to repel the P1 chain further out of the S1 pocket (Figure 5c). This is in line with data of Heinemeyer et al. who showed decreased conversion of Cbz-Gly-Gly-Leu-4-nitroanalide in Ala49Val mutated β5 subunit in the Yeast proteasome. In our in silico studies, the side chain of Thr21 does not seem to be directly involved in binding of the suc-LLVY-AMC substrate, although this Thr residue is in close proximity to the peptide backbone of the substrate (Figure 5a). Minor conformational changes may therefore result in a hydrogen bond between the Thr21 side chain and the backbone of LLVY. Therefore, the replacement of Thr by Ala may also hamper substrate binding (data not shown).

Introduction of the Ala49Thr mutation leads again to changes in the S1 pocket. As in case of BTZ, Thr49 can hamper P1 access for suc-LLVY-AMC to the specificity pocket (Figure 5d). Addition of the Met45Ile mutation further pushes the P1 group further out of the S1 pocket (Figure 5e). In conclusion, the 3D structural analysis of suc-LLVY-AMC binding to β5 subunit suggests that the experimentally observed mutations negatively affect the substrate’s affinity to the binding pocket.

Residual β5 proteasomal activity in BTZ-resistant cells

The in silico 3D modeling analysis of BTZ and suc-LLVY-AMC in complex with the mutated β5 subunit suggests that the observed mutations compromise BTZ as well as suc-LLVY-AMC binding. To investigate the binding capacity of the BTZ pocket in vitro, native gel electrophoresis was performed with equal amounts of protein extracts, followed by in gel β5 activity assay using the suc-LLVY-AMC substrate, as described previously. Figure 6a shows the effect of BTZ on in gel activity harbored by the core particle (CP) of the
Mutations in β5 subunit impair bortezomib binding

A

β5

Thr21

Cys52

Met45

B

Phe52

C

Phe52

Thr49

d

Thr49

E

Thr49

Ile45

Figure 5. In silico 3D-modeling analysis of mutations in β5 subunit shows decreased suc-LLVY-AMC binding capacity (A) Structural prediction of the active site of WT Bos Taurus β5 subunit in complex with suc-LLVY-AMC, compared to (B) Cys52Phe mutant, (C) Cys52Phe and Ala49Val double mutant, (D) Ala49Thr mutant, and (E) Ala49Thr and Met45Ile double mutant. Suc-LLVY-AMC is depicted in dark orange, mutations are highlighted in bright green and the catalytic active Thr1 is highlighted bright purple. Heavy atoms in hydrogen bonds are connected by dotted grey lines.
proteasome in parental CEM and 8226 cells and their BTZ-resistant sublines. For parental CEM and 8226 cells, a steep dose-response effect is noted with a marked inhibition of *in gel* proteasome activity at 7 nM BTZ and complete inhibition at 25 nM BTZ. For low level (BTZ7) and high level (BTZ100/200) BTZ-resistant cells, exposure to BTZ confers a less efficient inhibition upon *in gel* proteasome activity assay than in parental cells.

Since a significant loss of function of proteasome activity would lead to accumulation of ubiquitinated proteins, total protein ubiquitination levels were investigated in the BTZ resistant cells. Figure 6b compares the SDS-PAGE-resolved ubiquitinated proteins of untreated cells with 24h of treatment with BTZ. For parental cells, both low dose BTZ concentrations (i.e. 7 nM; 3-4 fold IC₅₀) and higher BTZ concentrations (25 nM) were used. For each cell line experiments were performed either in the absence of drug (parental cells), at their selective BTZ concentrations (7 nM for 8226/BTZ7 and CEM/BTZ7, 100 nM for 8226/BTZ100 and 200 nM for CEM/BTZ200), as well as BTZ concentrations of at least 2.5-fold beyond selective concentrations. Both parental CEM/WT and 8226/WT

![Figure 6](image_url)

*Figure 6. Effect of BTZ on direct and indirect proteasomal activity in WT and BTZ-resistant cells (A)*

Direct proteasomal activity in crude cell extracts of parental and BTZ-resistant CEM and 8226 cell lines as measured after native gel electrophoresis by analysis of Suc-LLVY-AMC conversion. Samples were harvested after 24h incubation with the indicated concentrations of BTZ. Inhibition by BTZ of *in gel* activity harboured by the core particle (CP) of the proteasome was determined (see arrow). Note: the higher molecular weight band represents the 20S proteasome core particle (CP) in complex with the regulatory 19S particle (RP).

*Figure 6. Accumulation of ubiquinated proteins in parental and BTZ-resistant CEM and 8226 lines after 24h incubation with the indicated concentrations of BTZ. For each cell line, experiments were performed in the absence of drug (parental cells), at their selective BTZ concentrations (7 nM for 8226/BTZ7 and CEM/BTZ7, 100 nM for 8226/BTZ100 and 200 nM for CEM/BTZ200), and BTZ concentrations of at least 2.5-fold above the selective BTZ concentrations.*
cells showed a dose-dependent increase of polyubiquitinated proteins upon exposure to BTZ. In contrast, BTZ-resistant cells did not show an accumulation of polyubiquitinated proteins at their selective drug concentrations of 100 and 200 nM BTZ, respectively. However, at very high concentrations (i.e. 500 nM), BTZ was able to induce the accumulation of ubiquitinated proteins, indicating that the ubiquitination machinery in BTZ resistant cells was still capable of responding by accumulation of ubiquitinated proteins when mutated β5-subunit-associated proteasome activity is fully blocked at high BTZ concentrations.

DISCUSSION

This study provides several lines of evidence for the involvement of multiple mutations in the β5 subunit of the proteasome in BTZ resistance in different hematological malignancy cell line models: (1) Multiple mutations in the BTZ-binding pocket of the β5 subunit were identified which mapped to a mutation cluster region in the established catalytic site, (2) We obtained 3D modeling evidence to support decreased BTZ affinity to the mutant β5 subunits, (3) The β5 proteasomal subunit was substantially overexpressed in BTZ-resistant cells, (4) A near complete substitution of the immune form of the β5 subunit by the constitutive mutated form, occurred in BTZ-resistant 8226 cells, (5) BTZ-resistant cells displayed a marked cross-resistance to proteasome inhibitors which selectively target β-subunits, but not the α7 subunit, (6) Finally, BTZ-resistant cells showed sustained overall proteasomal activity indicated by decreased accumulation of ubiquitinated proteins after exposure to BTZ.

Mutations in cellular enzymes targeted by chemotherapeutic drugs is a well established modality of drug resistance.1-3 This is also the case with BTZ resistance; we and others have reported mutations in the β5 subunit in pre-clinical BTZ-resistant cell line models.1,3,7,39 In our current study, we identified 4 novel mutations, all residing within the BTZ-binding pocket, and cluster with the previously reported G322A (Ala49Thr)1,7,39 and C323T (Ala49Val)3 mutations. The BTZ binding pocket, particularly the S1 pocket, proved to be a "hot spot" for mutations associated with BTZ resistance. In silico analysis of these amino acid substitutions revealed that Ala49Thr, Ala49Val and Thr21Ala are directly involved in BTZ-binding and therefore presumably result in decreased BTZ-binding affinity via loss of hydrogen bonds between BTZ and the β5 subunit, and by hampering accessibility to the S1 specificity pocket. The Cys52Phe mutation was found to cause repulsion of BTZ's P1 side chain out of the S1 pocket. The Met45lle and Met45Val substitutions were not found to directly interact with BTZ. However, Met45 resides in the S1 specificity pocket and contributes to its specificity. Moreover, upon binding, Met45 is known to undergo a conformational change and shifts the direction of its side
chain towards Cys52 vicinity. Alterations in Met45 might therefore compromise BTZ binding as well. It is not clear how the Thr21Ala is lost and replaced by a Ala49Thr in the 8226 cells upon additional selective pressure. It has been established in drug resistant cancer cells that the mutated gene can revert to WT by an additional mutation event. Loss of a mutation can in general also be explained by loss of heterozygosity (losing the mutated gene) or oligoclonality (disappearance of one subclone and emergence of another subclone with a different mutation). Which one of the processes have occurred in this BTZ-resistant cell line remains to be elucidated. Together, our in silico modeling data suggest that all identified mutations can decrease the affinity of the β5 binding pocket to BTZ.

Since the S1 pocket is also involved in substrate binding, we determined the influence of the mutations in the β5-subunit on suc-LLVY-AMC substrate binding. Using the crystal structure of the proteasome in complex with epoxomicin, which has structural similarity to the backbone of the suc-LLVY-AMC substrate, in silico structural prediction of the β5 subunit in complex with suc-LLVY-AMC was performed, suggesting decreased binding affinity of this substrate in the mutant β5 in BTZ-resistant cells. These in silico results were confirmed using β5 activity assay after native gel electrophoresis, which showed a profound decreased conversion of suc-LLVY-AMC in 8226/BTZ100 protein extract, although the β5 subunit was heavily overexpressed. Similar results were obtained with protein extracts from CEM/BTZ200 cells, albeit to a lesser extent. These cumulative findings suggest that cells harboring a mutant β5 subunit have impaired proteasome activity, relative to parental cells. Therefore BTZ-resistant cells must compensate for this impaired activity via upregulation of the β5 subunit. Consistent with this notion is the observation that in cells harboring homozygous β5 subunit mutations, upregulation of the β5 subunit was even more pronounced when compared to cells harboring heterozygous mutations. These findings underline the tight relationship between impaired proteasome activity and subunit overexpression. This compensation of impaired catalytic activity by subunit upregulation was clearly shown in both 8226/100 and CEM/BTZ200 cells, although the mechanism is different. It is noteworthy that CEM/BTZ cells upregulate both the constitutive and immune forms of the proteasome. Therefore, it is likely that the wild type β5 and β5i are responsible for the residual proteasome activity as determined in our native gel activity analysis with protein extracts from CEM/BTZ200 cells. In contrast, 8226/BTZ100 cells show a near complete substitution of the β5i subunit by the homozygous mutant constitutive β5 subunit, thereby resulting in almost no detectable in-gel activity. The residual activity detected is most likely mediated by the low residual expression of β5i subunit.

Since the complete loss of proteasome function is not compatible with life, we wondered whether or not the overall proteolytic activity of the proteasome remains intact. In BTZ-resistant cells, upon exposure to selective BTZ concentrations, inhibition of protea-
some activity is incomplete, hence sustained residual activity may still facilitate protein degradation without accumulation of ubiquitinated proteins. Only upon exposure to BTZ concentrations that are 2.5-5 fold higher than the selective concentrations of BTZ, proteasome activity is compromised, hence leading to accumulation of ubiquitinated proteins similar as observed for parental cells of low dose BTZ. These results indicate that the ubiquitination machinery remained intact in the BTZ resistant cells.

Interestingly, BTZ-resistant 8226 cells revealed a near complete shift of the β5i subunit to the constitutive β5 subunit. A similar shift in β5 and β5i expression profiles was previously reported by our laboratory for BTZ-resistant THP-1 AML cells. These observations could be consistent with the notion that up-regulation of a mutant constitutive β5 subunit in conjunction with a down-regulation of a non-mutated, and thus BTZ-inhibitable β5i subunit, would set the stage for conferring the highest level of BTZ-resistance.

BTZ-resistant cells show a marked cross-resistance towards proteasome inhibitors which target the β5 subunit (Table 1). Since BTZ-resistant cells retain full sensitivity to the α7 proteasome inhibitor 5AHQ, their propensity to die from proteasome inhibition with inhibitors that target other proteasomal subunits remains fully intact. This latter finding bears an important implication for the successful treatment of such BTZ-resistant tumors with agents like 5AHQ that do not target the structurally altered β5 subunit. Based on these findings, we propose a model in which acquired mutations in the β5 subunit attenuate BTZ binding and inevitably substrate binding, although the BTZ binding appears to be affected to a greater extent than natural substrates. Therefore, BTZ resistant cells must rely on the upregulation of mutant β5 subunit to overcome this decreased proteasome activity, and thereby retain the crucial overall protein turnover function. This model is in accord with our previously published THP1/BTZ100 cells showing upregulation of the mutant β5 subunit without increment in β5 proteasomal activity. In addition, no accumulation of ubiquitinated proteins was observed after exposing the THP/BTZ100 to BTZ. Consistently, Ri et al. also found in their BTZ resistant myeloma cell lines an Ala49Thr substitution and reduced accumulation of ubiquitinated proteins after 24h. In contrast to our data, they showed increased proteasome activity in their BTZ resistant lines harboring the Ala49Thr substitution. Likewise, Lu et al. showed increased proteasome activity of the β5 mutated proteasome. This difference might be explained by the specific chymotrypsin-like activity assay used; we used an in gel activity assay versus a whole cell lysate or intact cell assay. Moreover, 8226/BTZ100 cells expresses the mutant β5 subunit with only a minor β5i expression, which indicates that the mutated β5 subunit is solely responsible for the observed chymotrypsin-like activity in 8226/BTZ100 cells.

Given the heterogeneity of the patterns of clinical resistance to BTZ in MM, the molecular background of BTZ resistance may have a multifactorial basis. In the relapsed/refractory setting, the findings of the APEX study support the existence of inherent drug
Chapter 5

resistance as reflected by the 50% of patients that either lacked any response or displayed a progressive disease\(^{42}\). However, there is also convincing evidence for acquired BTZ-resistance from clinical practice. Specifically, BTZ treatment of patients experienced progressive disease during BTZ treatment and BTZ-based re-treatment as second line therapy is known to be less efficient compared to BTZ treatment of patients who were BTZ-naïve.\(^{43,44}\) These observations may support the hypothesis of the emergence of BTZ resistant clones upon proteasome inhibitor-based drug therapy and hence supported clonal selection following cycles of retreatment. Such post drug treatment specimens were not available for analysis in this study, since progressive disease is generally defined by an increase in M protein levels in combination with CRAB criteria (elevated Calcium, Renal failure, Anemia and Bone lesions), without the need for bone marrow analysis. Notwithstanding this fact, we screened for \textit{PSMB5} mutations in 14 bone marrow samples of MM patients at diagnosis before treatment with BTZ. Although 2 patients were found to be clinically refractory to treatment, no \textit{PSMB5} mutations were identified (results not shown). Moreover, other research groups also sequenced the \textit{PSMB5} gene in small groups of MM patients before BTZ treatment and did not detect mutations.\(^7,45\) Since data from the present study suggest that \textit{PSMB5} mutations are induced and/or selected during exposure to BTZ, collection of samples during treatment is warranted as part of a prospective study design. This is underscored by the recent publication of Shaughnessy et al.\(^{46}\) who provide evidence for a 48-hr post-BTZ gene expression profile of 80 genes which significantly overlaps with proteasome pathway-related genes including \textit{PSMD4}. Moreover, this profile was correlated with shorter progression free survival and overall survival.

It was of interest to note that \textit{PSMB5} mutations emerged more rapidly during in vitro BTZ exposure in leukemia cells of T-cell origin (CCRF-CEM cells) than in 8226 MM cells. It remains to be determined whether or not this is representative of the clinical setting. In fact, this will be investigated as part of an ongoing study that our laboratory is coordinating for childhood relapsed ALL treated with BTZ-containing chemotherapy. For this purpose we have set up and validated a sensitive high resolution melting analysis method for detecting each of the PSMB5 mutations identified in the current study.

In conclusion, our data show that prolonged in vitro exposure of cells of hematologic malignancies to BTZ, leads to mutations in the PMSB5 gene that result in amino acid substitutions residing in, or in close proximity to the BTZ binding pocket in the \(\beta5\) subunit. We provide evidence that support the model that alterations in the BTZ pocket result in decreased BTZ binding, hence forcing the mutant cells to compensate for this impaired catalytic activity by upregulation of the \(\beta5\) subunit. These results may have important clinical implications; we show that second generation proteasome inhibitors directed against \(\alpha7\) subunits can overcome this BTZ-resistance in cells harboring \(\beta5\) subunit mutations, hence offering a potential future treatment modality.
Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Conflicts of interest
The authors declare no conflict of interest.

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REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Antibodies and drugs
Proteasomal subunit antibodies and peptide substrates (Suc-LLVY-AMC) were obtained from Biomol-Enzo (Farmingdale, NY, USA). In addition, we purchased β-actin (clone c4) from Boehringer Mannheim (Almere, The Netherlands), α-Tubulin (B-7) and Ubiquitin (P4D1) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the IRDye infrared secondary labeled antibodies from LI-COR Biosciences (Lincoln, NE, USA).

BTZ was kindly provided by Millennium Pharmaceuticals (Cambridge, MA, USA). The proteasome-inhibitors MG132 (Z-Leu-Leu-Leucinal) and MG262 (Z-Leu-Leu-Leu-boronoate) were purchased from Calbiochem/ Merck (Nottingham, UK). The cytotoxic peptide 4A6 (Ac-Thr(tBu)-His(Bzl)-Thr(Bzl)-Nle-Glu(OtBu)-Gly-Bza) was synthesized as described previously. 5-Amino-8-Hydroxyquinole (5AHQ) was provided by Dr A.D. Schimmer (Toronto, Canada). The epoxyketone-based proteasome inhibitors Carfilzomib (CFZ, formerly PR-171) ONX 0912 (formerly PR047) and ONX 0914 (formerly PR957) were provided by Onyx Pharmaceuticals (CA, USA).

Cell growth inhibition assay

In vitro drug sensitivity was determined using the 4-day MTT cytotoxicity assay, as described previously. Prior to these experiments, bortezomib-resistant cells were transferred in bortezomib-free medium for at least 2-4 days. The IC50 value was defined as the drug concentration needed to inhibit 50% of the cell growth compared to growth of the untreated control cells.

Protein expression
Cell pellets of 5 x10⁶ cells were snap frozen in liquid nitrogen and subsequently resuspended in 100 μl lysis buffer (PBS containing 1% Igepal CA-630 (Sigma-Aldrich Zwijndrecht, NL) and Complete™ protease inhibitor cocktail (Boehringer Mannheim, Almere, NL) for 45 min at 4°C. Lysates were clarified by microcentrifugation at 14 000g during 5 min. Next, Laemli’s sample buffer (Biorad, Veenendaal, NL) supplemented with 50 μl of β-mercaptoethanol/1 ml was added (lysis buffer: Laemli buffer =2:1). Lysates were heated at 95°C for 5 min, separated by dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5–12.5%) and electroblotted (30 V, 12 h) onto Immobilon-FL PVDF membranes (Millipore, Billerica, MA, USA). Next, membranes were washed with PBS, incubated for 1 h in Odyssey blocking buffer (Li-cor biosciences, Lincoln, NE, USA) and incubated with the primary antibody for 1h at RT. Thereafter, membranes were washed with PBS-Tween and incubated with infrared labeled secondary antibody for 1 hour at RT. After washing four times in PBS, proteins were visualized using the Odyssey® Infrared Imaging System (Li-cor biosciences, Nebraska, USA).
Protein levels of the catalytic proteasomal subunits were also quantified using a sandwich ELISA method as described previously by Parlati et al. 31

Native gel electrophoresis
Characterization of the proteasome (detection of core/regulatory particles and suc-Leu-Leu-Val-Tyr-AMC hydrolysis activity), in WT and BTZ-resistant cells by native gel electrophoresis was performed essentially as described by Elsasser et al. 30 using acid washed glass beads (Sigma) for the preparation of lysates. Subsequent western blot analysis was performed as described above.

Quantitative RT-PCR and cDNA synthesis of proteasome subunits
After RNA isolation by the TRIzol method (Invitrogen), cDNA was synthesized using RT buffer (Invitrogen), containing 5mM DTT (Invitrogen), 2mM dNTP(Roche), pdN6 96ug/ml (Roche), 0.75U/ul M-MLV (Invitrogen) and 2U/ul RNasin (HT Biotechnology Ltd., Cambridge, UK). mRNA expression levels of proteasome subunits PSMB5 (β5), PSMB6 (β1), PSMB7 (β2) and the endogenous housekeeping gene β-glucuronidase (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). Primers-probe combinations and concentrations used for the quantitative real-time PCR. All probes were labeled with 5'-FAM and 3'-'BHQ1 as a reporter. Primers and probes were designed using Primer Express software (Applied Biosystems) as follows: PSMB5 forward (50 nM): CTTCAAGTTCCGCCATGGA; PSMB5 reverse (300 nM): CCGTCTGGGAGGCAA TGTA; PSMB5 probe (200 nM): TTGCAGCTGACTC CAGGGCTA - CAGC; PSMB6 forward (300 nM): AGGCATGACCAAGGAAGAGTGT; PSMB6 reverse (50 nM): GAGCCATCCCGCTCCAT; PSMB6 probe (200 nM): TGCAATTCACTGCCAAT GCTCTCGC; PSMB7 forward (300 nM): TCGGTGTA TGCTCCACCAGTT; PSMB7 reverse (50 nM): GCAGGACGCTCCAAGAC; PSMB7 probe (200 nM): TTCTTCTT TGATAACTGCCGAG- GAATGC; GUS forward (300 nM): GAAATATGTGGTTG GAGAGCTCATT; GUS reverse (300 nM): CCGA GTGAAGATCCCCTTTTTA; GUS probe (200 nM): CCAGACCTCTCGGTGAGC TGTTC. Real-time PCR was performed in a total reaction volume of 50 μl containing TaqMan buffer A (Applied Biosystems), 4 mM MgCl2, 0.25 μM 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.49 The Ct of the target gene is normalized to the GUS PCR 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%.
Sequence analysis

DNA was isolated from wild-type (WT) and BTZ resistant cells using a Qia amp DNA blood mini kit (250) (Qiagen, Valencia, CA, USA). Subsequently, the region of interest of the *PSMB5*, *PSMB6* or *PSMB7* gene was amplified by PCR. The primers were designed using Vector NTI (Invitrogen) software and depicted in supplementary table S1. PCR products were directly sequenced by dideoxy chain-termination method using a kit ABI PrismTM BigDye Terminator (Perkin Elmer, Foster City, CA, USA) and analyzed by an autosequencer ABI Prism Genetic Analyser 3100 automatic DNA sequencer (Perkin Elmer).
Supplementary Table S1. Sequencing primers

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<th>Exon</th>
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<th>Reverse</th>
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