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Probing proteasome activity and function : cancer diagnostics and mechanism of antigen processing

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Chapter 2.2

**Profiling patient response to
marizomib therapy**

Profiling patient response to marizomib therapy

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Marizomib (NPI-0052; Salinosporamide A) is a irreversible proteasome inhibitor isolated from the marine actinomycete *Salinispora tropica* that has demonstrated activity in a wide range of preclinical tumor models. Clinical trials with marizomib are currently ongoing, enabling a detailed study of patient response to marizomib treatment. In the present study, we used a fluorescent proteasome activity probe to profile proteasome inhibition in both packed whole blood (PWB) and peripheral blood mononuclear cells (PBMCs) from patients on marizomib therapy. We show that PWB, which contains >98% red blood cells in which immunoproteasome is not expressed, responds differently to proteasome inhibitors and recovers slower from proteasome inhibition compared to PBMCs. In addition, we demonstrate that proteasome activity levels prior to treatment differ between patients, and that this initial proteasome activity level is an important determinant of the sensitivity of cells to proteasome inhibitors. Furthermore, we show that PBMCs are able to rapidly change their proteasomal subunit composition in response to marizomib treatment, which seems to account for a quick recovery, suggesting that this ability may mediate sensitivity of tumor cells to proteasome inhibition. Together, these data indicate that fluorescent proteasome probes can be used to profile patient blood samples and that they may enable prediction of patient responses to proteasome inhibitors, which can contribute to a more personalized proteasome inhibition strategy to improve the therapeutic potential of this class of drugs and finally clinical outcome of proteasome targeted therapy.

INTRODUCTION

Proteasome inhibition has emerged as a powerful approach for the treatment of cancer, as evidenced by the clinical success of the proteasome inhibitor bortezomib (Figure 1).¹ Bortezomib has been approved for the treatment of relapsed and newly diagnosed multiple myeloma (MM)²⁻⁴ and mantle cell lymphoma⁵ and in combination with PEGylated doxorubicin for the treatment of relapsed MM.^{6,7} Bort-

ezomib treatment is associated with substantial side effects, and the occurrence of both primary and secondary resistance has been reported.⁸ Consequently, there is an ongoing search for novel proteasome inhibitors that are able to overcome bortezomib resistance and that are better tolerated to increase their therapeutic potential. Several second-generation proteasome inhibitors have entered clinical trials, including marizomib (NPI-0052; Salinosporamide A)⁹ (Figure 1), CEP-18770,¹⁰

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and Carfilzomib (PR-171).¹¹

The proteasome is a large threonine protease complex responsible for the degradation of redundant and misfolded proteins and for the turnover of regulatory proteins that control a wide range of cellular processes, including cell cycle regulation, apoptosis and proliferation.^{12,13} Mammalian 26S proteasomes consist of a 20S catalytic core, complexed at one or both ends with 19S regulatory caps. The 20S core is composed of four stacked rings of seven subunits each and has an overall architecture of $\alpha(1-7)\beta(1-7)\beta(1-7)\alpha(1-7)$. The two outer α -rings stabilize the complex and serve as docking stations for the binding of regulatory caps, while the catalytic activity resides within the two inner β -rings. Three constitutive β -subunits, termed $\beta1$, $\beta2$ and $\beta5$, provide the proteasomal caspase-like, tryptic and chymotryptic activity, respectively. In lymphoid tissues and in cells exposed to $\text{INF-}\gamma$ and $\text{TNF-}\alpha$, these subunits are replaced by their immunoproteasomal counterparts $\beta1i$, $\beta2i$ and $\beta5i$ to form the immunoproteasome,¹⁴ which is thought to favor the production of antigenic peptides,¹⁵ while the existence of mixed-type hybrid proteasomes has also been reported.^{16,17}

Marizomib is an orally available proteasome inhibitor isolated from the marine actinomycete *Salinispora tropica*.⁹ Marizomib irreversibly inhibits the proteasome via a two-step mechanism. In an initial step an ester is formed between the catalytic Thr1 O^v of the proteasome and the carbonyl derived from the β -lactone ring. Subsequent chlorine substitution then gives rise to a cyclic ether.¹⁸ In contrast to bortezomib which targets the $\beta5$ and $\beta1$ subunits, marizomib has been shown to inhibit all constitutive and immunoproteasome subunits.⁹ Recent studies have shown that inhibition of multiple activities is a prerequisite for a significant inhibition of protein degradation, depending on the protein that is

degraded,¹⁹ suggesting that blocking all three activities may be therapeutically advantageous. Marizomib showed efficacy as a single agent or in combination with conventional chemotherapy in a variety of preclinical solid tumor and hematologic tumor models²⁰⁻²² and was able to overcome bortezomib resistance in tumor cells purified from MM patients that had relapsed from prior therapies including bortezomib treatment.⁹ Interestingly, combination treatment with bortezomib and marizomib both *in vitro* and *in vivo* induced synergistic cytotoxicity in MM and Waldenstrom's macroglobulinemia.^{23,24} Marizomib is currently being evaluated in phase I clinical trials for the treatment of both solid and hematologic tumors.

Proteasome activity is traditionally measured using fluorogenic substrates for the different catalytic activities.²⁵ The use of recently developed chemical proteasome activity reporters provides an alternative method for assaying proteasome activity in a wide range of tissues.²⁶⁻²⁸ In this study, we used a fluorescent proteasome activity reporter²⁷ to compare the inhibitory profiles of marizomib and bortezomib in rat blood samples, and established that this probe can be used to accurately profile both packed whole blood (PWB) and peripheral blood mononuclear cell (PBMC) samples. In addition, we developed a protocol that enables the use of this fluorescent probe to profile blood samples from patients that are treated with proteasome inhibitors. The data presented here give for the first time insight into patient response to and recovery from marizomib treatment and show that both the initial proteasome activity in cells and the ability of cells to change the composition of newly synthesized proteasomes may mediate sensitivity to this novel class of drugs. In addition, we show that immunoproteasome is not expressed in PWB, which contains >98% red blood cells. As red

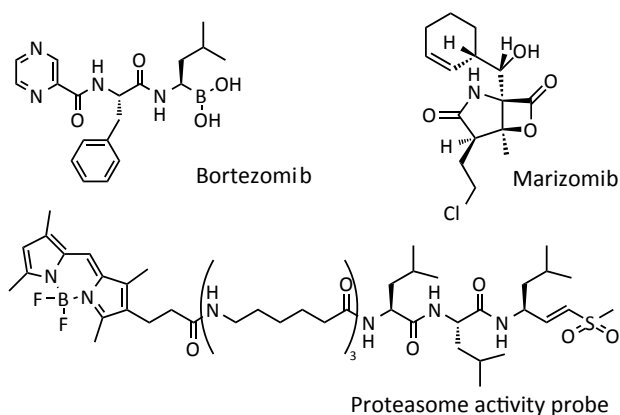


Figure 1 | Structures of bortezomib, marizomib, and proteasome activity probe 1.

blood cells have a relatively high, micromolar intracellular proteasome concentration,²⁵ they are thought to sequester intravenously administered proteasome inhibitors *in vivo*.²⁹ Consequently, proteasome inhibitors likely saturate proteasomes in red blood cells before peripheral sites can be reached. Specific immunoproteasome inhibitors, however, may saturate blood proteasome at relatively low concentrations, which may result in higher inhibitor concentrations in tumor cells. This suggests that immunoproteasome inhibitors may in future provide a good alternative for tumors that have high intrinsic immunoproteasome activity.

RESULTS

Profiling marizomib and bortezomib in rat blood samples *ex vivo*

To profile the effects of proteasome inhibitors on blood proteasome in both rats and patients *ex vivo*, fluorescent proteasome activity reporter **1** (Me₄BodipyFL-Ahx₃Leu₃VS, described in chapter 2.1) was used.²⁷ Probe **1** consists of a proteasome-targeting motif, a vinyl sulfone reactive group that covalently reacts with the N-terminal threonine of all active subunits and a fluorescent tag (Figure 1).

This tag allows the use of an SDS-PAGE based assay to visualize proteasome (subunit) labeling by scanning the gel for fluorescence emission. Prior inhibition of proteasome subunits prevents probe binding, which results in the disappearance of fluorescent signal. Therefore, the measured fluorescence intensity directly correlates to the activity of a labeled β -subunit. To study whether proteasome inhibition could be reliably measured in blood samples using probe **1**, the inhibition and recovery patterns of bortezomib and marizomib were evaluated in rat blood samples. Rats ($n=15/\text{group}$) were treated with vehicle, 0.15 mg/kg bortezomib or 0.05 or 0.10 mg/kg marizomib (which will be referred to as marizomib low and marizomib high treatment, respectively). Blood samples were taken from 3 rats per dosing group per time point (1.5 h, 24 h, 48 h 72 h or 168 h after injection), packed whole blood (PWB) or PBMC lysates were obtained, and lysates were incubated with probe **1**. Proteins were then separated on SDS-PAGE, and the resulting gel was scanned for fluorescence emission. Figure 2A shows representative gel images of labeled PWB and PBMC samples.

Red blood cells (RBCs), which do not express immunoproteasome, form the majority of

PWB (>99%), and as a result mainly constitutive proteasome subunits were labeled in PWB samples (Figure 2A). In PBMCs, which consist mainly of white blood cells, both the constitutive $\beta 1$, $\beta 2$, and $\beta 5$ subunits and the immunoproteasomal $\beta 2i$ and $\beta 1i$ subunits were clearly labeled (Figure 2A). In both PBMC and PWB samples, no substantial differences were found between untreated and vehicle treated mice. The contribution of each subunit to total activity was constant, indicating that vehicle treatment did not influence proteasome activity (Supplementary Figure 1). Total proteasome activity differed somewhat between individual mice within treatment groups (data not shown). Upon treatment with marizomib or bortezomib, both total proteasome activity (Figure 2A; lanes 7-15) and the relative subunit labeling (Supplementary Figure 1) changed, indicating inhibition.

Marizomib and bortezomib have differential effects on PBMCs but not on PWB

To evaluate the effects of marizomib and bortezomib treatment on total proteasome activity, total fluorescence intensity in each sample was quantified, averaged per group, and plotted as percentage of untreated samples $\pm$ SEM (Figure 2B). In PBMCs a single dose of 0.05 mg/kg (low) or 0.10 mg/kg (high) marizomib reduced total proteasome activity to 20-30% residual activity, whereas a single dose of bortezomib (0.15 mg/kg) reduced total proteasome activity to 60%. Differences between marizomib high and bortezomib treatment remained significant up to 72 h after dosing, while marizomib low and bortezomib treatment had similar effects at time points 24 h to 168 h after dosing. Total proteasome activity recovered fully within one week for all treatment schedules. In PWB, all treatment regimes inhibited total proteasome activity almost completely. Recovery was hardly evident at time points up to 48 h, and one week

after dosing (168 h) only 50% of proteasome activity had recovered. Rats treated with a high dose of marizomib showed somewhat less recovery of total proteasome activity at 72 h and 168 h post dosing compared to bortezomib treated rats, but differences were not significant.

To study how individual subunits contributed to total proteasome activity, the fluorescence intensity of individual subunits was quantified, averaged per group, and plotted as the percentage of untreated samples $\pm$ SEM (Figure 2C). Since the $\beta 2$ and $\beta 2i$ subunits responded similarly to all treatments (data not shown), $\beta 2$ activity was defined as the sum of both bands. In PBMCs, the combined $\beta 5(i)/\beta 1$ activity was inhibited to a higher extent by marizomib high dose treatment compared to bortezomib treatment, while the $\beta 1i$ subunit was inhibited to a similar extent by both treatment regimes. The $\beta 2$ subunits were inhibited by marizomib, but not by bortezomib. These data suggest that the more potent and sustained inhibition of proteasome activity in marizomib high dose-treated rats compared to bortezomib treated rats is mediated by marizomib's ability to inhibit all proteasome activities. When bortezomib treatment was compared to marizomib low dose treatment schedules, inhibition of the $\beta 5(i)/\beta 1$ subunits was comparable, but differences were observed in inhibition of both the $\beta 2$ and $\beta 1i$ subunits. Inhibition of $\beta 2$ activities was found only in marizomib low treated rats, but not in bortezomib treated rats, as also previously reported.⁹ Inhibition of $\beta 1i$ activity was much more pronounced in bortezomib treated rats which recovered slowly, resulting in <50% recovery one week post-dosing. Together, these data suggest that the long-term effect of bortezomib and marizomib treatment on total proteasome activity was mediated via the inhibition of different sets of subunits. In PWB, differences between treatment regimes were

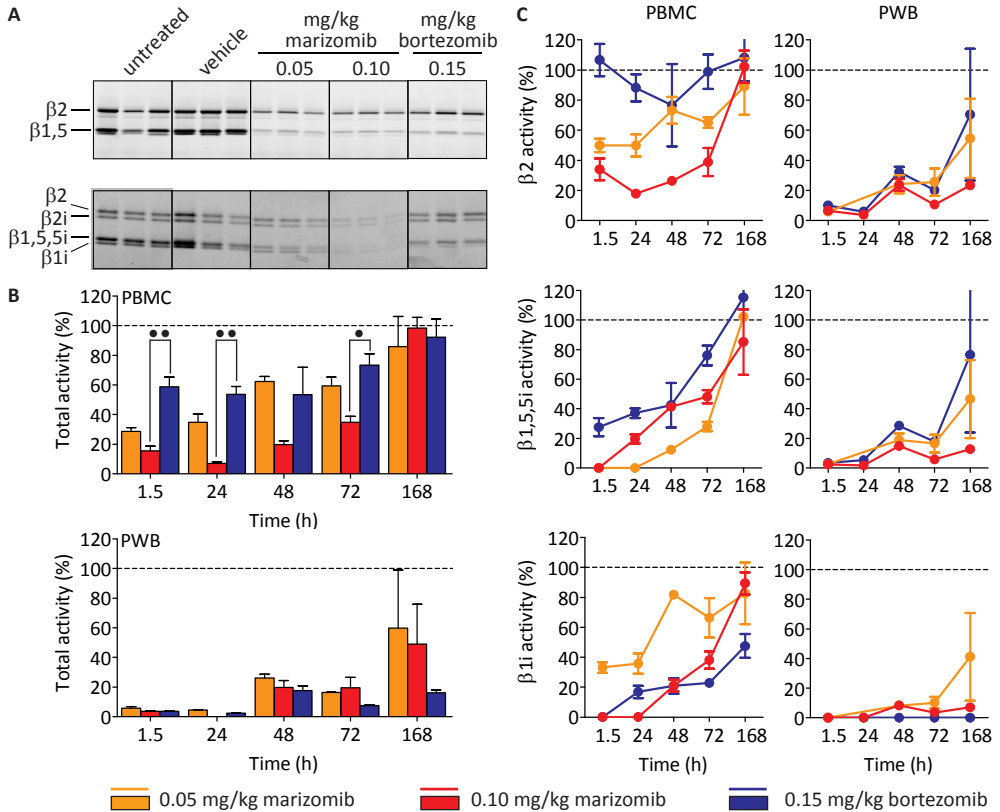


Figure 2 | Marizomib and bortezomib have different inhibition profiles in rat PBMC and PWB samples. Rats ($n=15$ /group; 3/time point per dosing group) received a single injection of 0.05 or 0.10 mg/kg marizomib, 0.15 mg/kg bortezomib or vehicle. PWB and PBMC samples were obtained at 1.5 h, 24 h, 48 h, 72 h or 168 h post dosing. Samples were lysed, followed by proteasome labeling with probe 1 and SDS-PAGE analysis. **A**) In-gel fluorescence measurements showing proteasome activity profiles in rat PWB and PBMC samples 1.5 h after treatment with marizomib or bortezomib. **B**) Quantification of total proteasome activity in rat PWB and PBMC samples after marizomib or bortezomib treatment for the indicated periods. Total proteasome activity was defined as the sum of all individual activities and was plotted as a percentage of activity in untreated rats. Error bars represent SEM. Data were analyzed by GraphPad Prism software. • $p < 0.05$; •• $p < 0.01$. **C**) Quantification of proteasome subunit activity in rat PWB and PBMC samples after marizomib or bortezomib treatment for the indicated time periods. Subunit activities were plotted as percentage of subunit activity in untreated rats. Error bars represent SEM.

largely absent compared to PBMC samples. Both marizomib and bortezomib inhibited all subunits, and recovery was also largely comparable between treatment schedules. The main difference was found at the level of the $\beta 1i$ activity, which recovered from marizomib

high or marizomib low treatment at a similar rate as the $\beta 2$ and $\beta 5(i)/\beta 1$ activity, but did not recover from bortezomib treatment. Since $\beta 1i$ activity was hardly present in PWB samples (see above), the effect of this difference on total proteasome activity was negligible.

Table 1 | Treatment regimes of patients #1 to #5.

Patient	Malignancy	Cycle 1	Subsequent cycles
#1	Prostate carcinoma	0.9 mg/m ²	0.7 mg/m ²
#2	Anorectal squamous carcinoma	0.9 mg/m ²	0.7 mg/m ²
#3	Hodgkin's lymphoma	0.8 mg/m ²	0.8 mg/m ²
#4	Colon carcinoma	0.8 mg/m ²	0.7 mg/m ²
#5	Non-Hodgkin's lymphoma	0.8 mg/m ²	0.7 mg/m ²

Remarkably, both bortezomib and marizomib treatment resulted in the disappearance of $\beta 2$ labeling by probe **1**, whereas bortezomib has been shown to somewhat enhance and not inhibit the activity of $\beta 2$ subunits in PWB using fluorogenic substrates.⁹ A similar disappearance of $\beta 2$ labeling after bortezomib treatment has been observed using probe **1** in cell lines *in vitro* and *ex vivo* in mouse tissues (see Chapter 2.1).

Probe 1 can profile proteasome activity in marizomib treated patients *ex vivo*

Having established that probe **1** can be used to assay the effects of proteasome inhibitors in both PBMC and PWB samples *ex vivo*, we next used probe **1** to evaluate the effect of marizomib treatment on patients suffering from different types of cancer (Table 1). Marizomib was administered intravenously at Day 1 (D1), Day 8 and Day 15 (D15) during 28-day treatment cycles (indicated with C1-C2). In the first treatment cycle, patients were dosed with concentrations of marizomib ranging from 0.55 mg/m² to 0.9 mg/m². Due to the occurrence of dose-limiting toxicities, marizomib concentrations were adjusted to 0.7 mg/m² or 0.8 mg/m² in subsequent cycles (Table 1). Blood was taken pre- and post-marizomib treatment; PBMCs and PWB samples were obtained and prepared in cell pellets. To study proteasome inhibition in PWB samples, PWB samples were lysed and incubated with probe **1**. Labeled subunits were separated by SDS-PAGE, and the resulting gels were scanned for

fluorescence emission. An example of such a gel is shown in Figure 3A. Analogous to results found in rat blood samples, only constitutive proteasome subunits were labeled in PWB. Marizomib treatment decreased the activity of both $\beta 2$ and $\beta 5/\beta 1$ subunits in PWB in all patients. Although in most patients some proteasome activity recovery was observed before the next dose of marizomib was given, proteasome activity never recovered to baseline levels in any of the patients (Figure 3A, pre treatment samples). These data are in accord with a previous report which suggested that PWB proteasome activity could be titrated with repeated dosing.²⁹

To validate that probe **1** could be used to accurately study the effects of proteasome inhibitors on patient blood samples, we compared probe **1** with the use of fluorogenic substrates for the different activities in PWB samples obtained at C1D1 (Cycle 1 Day 1) and C1D15 (Cycle 1 Day 15) post treatment (Figure 3B). For both assays, the residual activity of different subunits was plotted as a percentage of the baseline activity before the start of treatment. As the $\beta 1$ and $\beta 5$ subunits labeled by probe **1** were not completely resolved on gel, we averaged the $\beta 1$ and $\beta 5$ activity levels obtained using fluorogenic substrates to be able to compare the two methods. Results obtained with probe **1** were very similar to results found using fluorogenic substrates (Figure 3B). Both methods revealed a higher inhibition of the $\beta 1/\beta 5$ activity compared to the $\beta 2$ activity. In addition, both methods showed

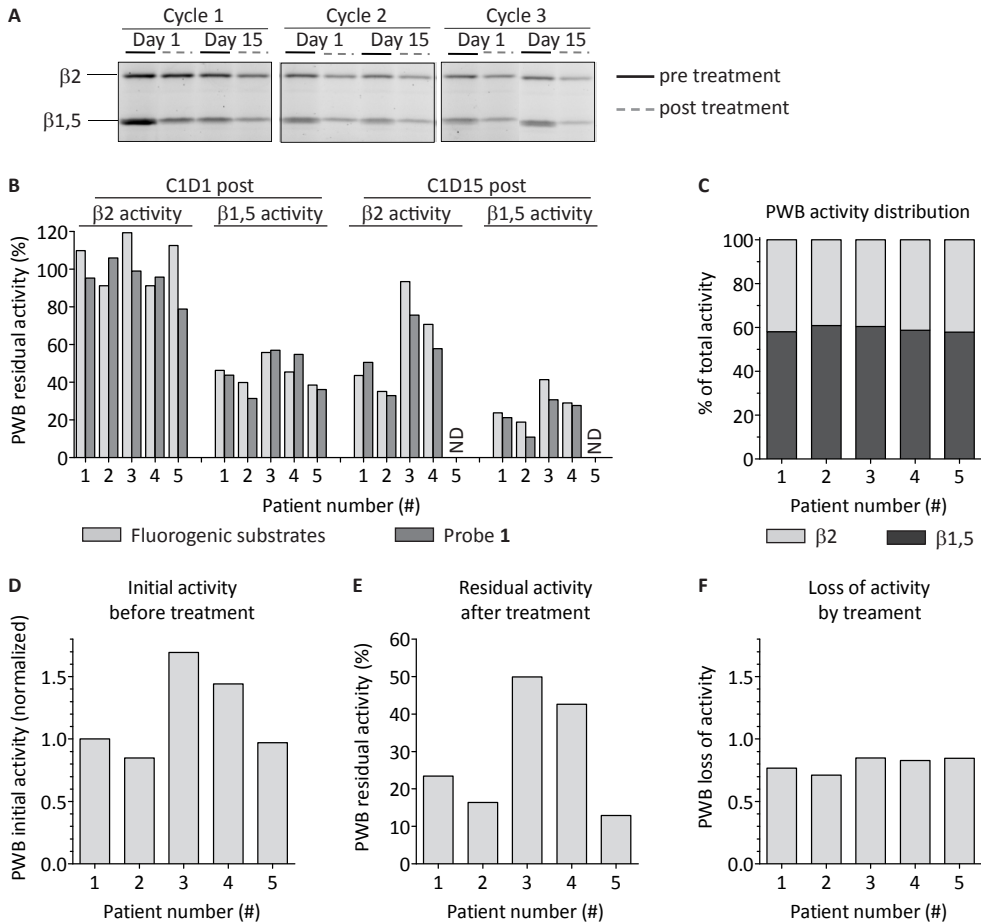


Figure 3 | Ex vivo profiling of proteasome activity in patient PWB samples. Patients with different malignancies (Table 1) were treated with varying doses of marizomib. Marizomib was administered at day 1, day 8, and day 15 in 28-day treatment cycles. Blood was taken pre and post dosing at days 1 and 15; PWB samples were prepared, lysed, and incubated with probe 1. Labeled proteasome subunits were separated using SDS-PAGE, and the resulting gels were scanned for fluorescence emission. **A**) In-gel fluorescence measurements showing proteasome activity profiles in PWB samples of patient #1 that were obtained during marizomib treatment. **B**) Comparison of residual proteasome activities in PWB samples from patients #1 to #5 obtained at Cycle 1 Day 1 (C1D1) and Cycle 1 Day 15 (C1D15) post treatment. Residual activities compared to baseline values were calculated using probe 1 or fluorogenic substrates. ND: not determined. **C**) Quantification of the relative contribution of the β2 and β5/β1 subunits to total baseline proteasome activity in patient PWB samples prior to marizomib treatment. **D**) Quantification of baseline total proteasome activity levels in PWB samples of patients #1 to #5 before the start of marizomib treatment. Baseline activities were normalized to patient #1. **E**) Quantification of residual total proteasome activity levels at Cycle 2 Day 15 (C2D15) post treatment compared to baseline proteasome activity levels in PWB samples obtained from patients #1 to #5. **F**) Quantification of the absolute amount of inhibited proteasome activity at C2D15 post treatment compared to baseline values in PWB samples of patients #1 to #5. Samples were normalized to baseline proteasome activity in patient #1.

that repeated dosing resulted a higher extent of inhibition (compare C1D15 to C1D1 in Figure 3B). Both methods also revealed that the inhibition in PWB was dose-dependent, especially at C1D15 post treatment. Patients #1 and #2 were treated with 0.9 mg/m² in the first treatment cycle, while patients #3, #4 and #5 received 0.8 mg/m² during the first treatment cycle. In accord, patients #3 and #4 showed higher residual β 2 and β 5 activities at C1D15 post treatment compared to patients #1 and #2 (for patient #5 no PWB sample was available at C1D15 post treatment). Together, these results indicate that probe 1 can be reliably used to assay proteasome activity in patient blood samples *ex vivo*.

Proteasome inhibition by marizomib differs between individual patients

We quantified proteasome inhibition as profiled using probe 1 in PWB samples of patients #1 to #5 (obtained during C1 and C2). In PWB baseline samples of different patients that were obtained before the start of marizomib treatment, identical β 2/ β 5,1 ratios (Figure 3C) with different total proteasome activity levels (Figure 3D) were observed. To compare inhibition profiles between patients, proteasome activity at C2D15 (Cycle 2 Day 15) post dosing was compared to baseline activity for patients #1 to #5. In accord with marizomib's inhibition profile, inhibition of the β 5 and β 1 subunits was approximately 2 fold higher than inhibition of the β 2 subunit in all patients (Supplementary Figure 2A). Total proteasome inhibition, however, differed substantially between patients, with residual proteasome activities ranging from 13% to 50% (Figure 3E). The residual proteasome activity correlated well to the initial proteasome activity in all patients. Patients with high initial proteasome activity also displayed high residual activity, while patients with low initial activity also showed low residual proteasome activ-

ity (compare Figures 3D and 3E). This suggested that all patients lost a similar absolute amount of proteasome activity as a result of marizomib administration. To investigate this, we subtracted the total fluorescence intensity at C2D15 post dosing from the baseline fluorescence intensity in each patient to calculate the loss of fluorescence intensity over the course of two treatment cycles (Figure 3F). As the total fluorescence intensity directly correlates to total proteasome activity in these samples, the loss of fluorescence directly correlates to the loss of proteasome activity. As can be seen from Figure 3F, a similar amount of proteasome activity was inhibited in all patients in PWB at the end of the second treatment cycle. Therefore, the residual proteasome activity in PWB after marizomib treatment is mainly determined by the initial amount of proteasome activity.

Initial proteasome activity determines PBMC response to marizomib

Next we investigated proteasome inhibition by marizomib in patient PBMCs. To this end, PBMCs were lysed and incubated with probe 1. Labeled subunits were separated by SDS-PAGE and the resulting gels were scanned for fluorescence emission. An example of such a gel is shown in Figure 4A. Analogous to results found in rat blood samples, both constitutive and immunoproteasome subunits were labeled in PBMCs (Figure 4A). In contrast to results found in PWB, the relative contribution of subunits to total proteasome activity differed between patients (Figure 4B). Diverse labeling patterns were observed, ranging from almost equal contribution of all subunits to total activity in patient #3, to subunits being responsible for over 50% (β 2i and β 1i subunits in patients #2 and #5, respectively) or less than 5% (β 2 in patients #1 and #5) of total proteasome activity. In PBMCs baseline samples of different patients, total proteasome

activity levels were variable (Figure 4C) and resembled initial proteasome activity levels in the corresponding PWB samples. In both types of samples, high initial proteasome activity was found in patients #3 and #4, patient #1 had intermediate activity, while the lowest activity was found in patients #2 and #5 (Figures 3D and 4C). Administration of marizomib decreased the activity of all subunits in all patients. In contrast to results found in PWB, total proteasome activity was largely restored to baseline levels before a new dose of marizomib was administered (Figure 4A).

We quantified proteasome inhibition and recovery in PBMC samples of patients #1 to #5 (obtained during C1 and C2). Since proteasome activity recovers between doses in PBMCs, the residual proteasome activity at C2D15 post treatment compared to baseline levels depended on the extent of recovery. However, each dose resulted in a similar reduction in proteasome activity in post treatment samples compared to the preceding pre treatment sample, independent of the extent of recovery in this pre treatment sample. Therefore, we first normalized the inhibition in each post treatment sample to the corresponding pre treatment sample, and plotted the average residual activity $\pm$ SEM. In PBMCs, the average residual proteasome activity differed between patients and ranged from 33% to 52% (Figure 4D). In addition, the average residual proteasome activity correlated well to the initial proteasome activity in all patients, as observed in PWB. In accord with results found in PWB, inhibition of the β 2,2i subunits in PBMCs was two fold lower compared to inhibition of the β 5,5i and β 1,1i subunits, with the exception of patient #5 in which all subunits were equally inhibited (Supplementary Figure 2B). Next, total fluorescence intensity in each post treatment sample was subtracted from the total fluorescence intensity in each corresponding pre treatment sample.

On average, a similar absolute amount of proteasome activity was inhibited in PBMCs as a result of each marizomib administration in all patients (Figure 4E). This suggests that the initial amount of proteasome activity in PBMCs is one of the factors determining the residual amount of proteasome activity after marizomib treatment.

Patient PBMCs change their proteasome composition in response to marizomib

Next, we studied the influence of recovery on proteasome inhibition in patient PBMCs samples. To take recovery effects into account, we calculated the residual activity at C2D15 post treatment compared to baseline activity before the start of treatment (Figure 4F, shaded bars). We compared these values to the average residual activity (Figure 4F, filled bars) in which recovery effects were not taken into account (as plotted in Figure 4D). In patient #1, activity recovered almost completely between doses and therefore little difference was observed between the average residual activity and the residual activity at C2D15 post treatment. In patient #4, activity recovered >100%, resulting in an increase in proteasome levels over time. As a consequence, the residual activity at C2D15 post treatment was higher compared to the average residual activity. In patients #2, #3 and #5, proteasome activity did not completely recover to baseline levels between doses, and therefore the residual proteasome activity at C2D15 post treatment was lower compared to the average residual activity. Together, these data suggest that the extent of activity recovery between administrations influences the efficacy of marizomib treatment over multiple treatment cycles.

To study recovery in individual patients in more detail, we quantified the relative contribution of individual subunits to total proteasome activity in PBMC samples from patients

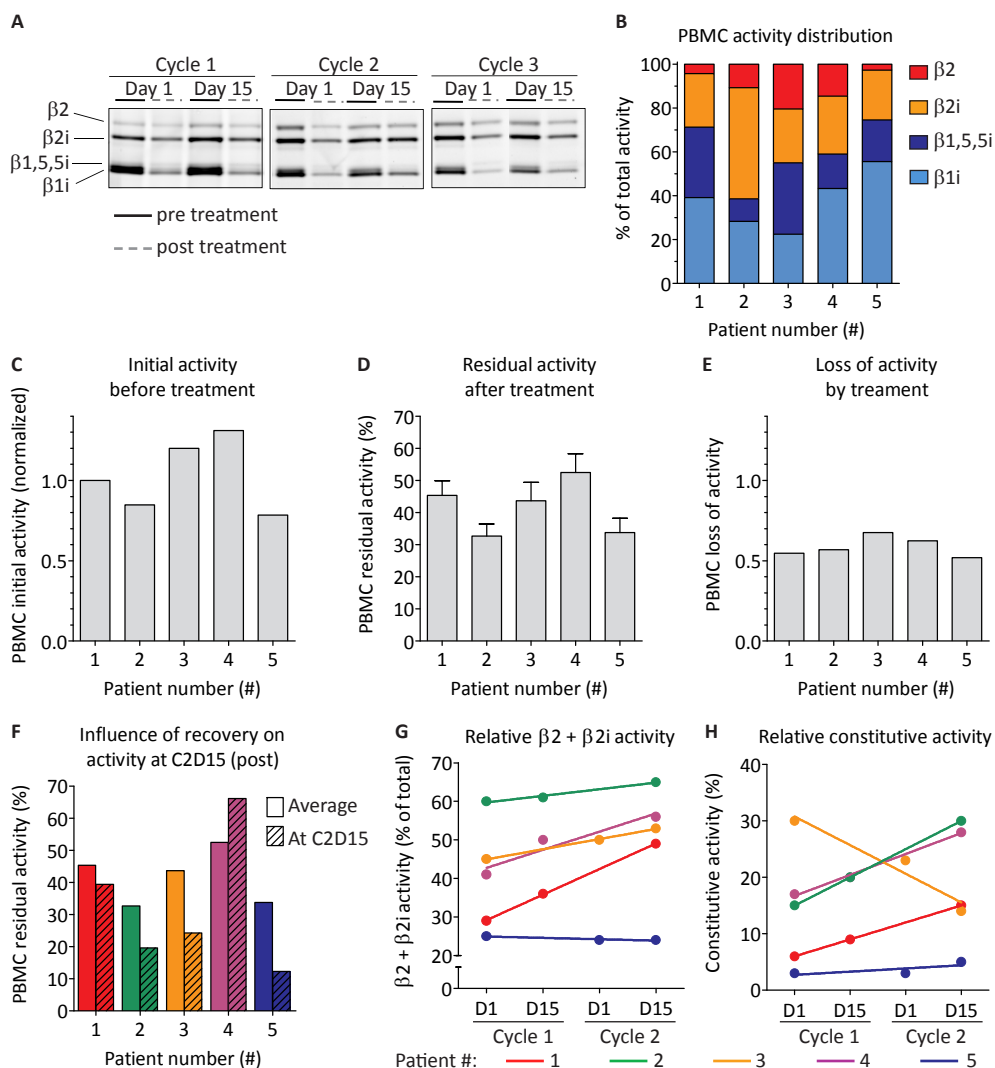


Figure 4 | Ex vivo profiling of proteasome activity in patient PBMC samples. Patients with different malignancies (Table 1) were treated with varying doses of marizomib. Marizomib was administered at day 1, day 8 and day 15 in 28-day treatment cycles. Blood was taken pre and post dosing at days 1 and 15 and PBMC samples were prepared, lysed and incubated with probe 1. Labeled proteasome subunits were separated using SDS-PAGE and the resulting gels were scanned for fluorescence emission. **A)** In-gel fluorescence measurements showing proteasome activity profiles in PBMC samples of patient #1 that were obtained during marizomib treatment. **B)** Quantification of the relative contribution of the β2, β2i, β1/5/5i and β1i subunits to total baseline proteasome activity in patient PBMC samples prior to marizomib treatment. **C)** Quantification of baseline total proteasome activity levels in PBMC samples of patients #1 to #5 before the start of marizomib treatment. Baseline activities were normalized to patient #1. **D)** Quantification of residual total proteasome activity levels post marizomib treatment in PBMC samples obtained from patients #1 to #5. Proteasome activity in each post treatment sample was normalized to the preceding pre treatment sample and

averaged over all administrations. Error bars represent SEM. **E)** Quantification of the absolute amount of proteasome activity that disappeared on average in PBMC samples of patients #1 to #5 after each administration of marizomib. Samples were normalized to baseline proteasome activity in patient #1. **F)** Quantification of the average residual proteasome activity after each administration in PBMCs obtained from patients #1 to #5 (solid bars). Quantification of the residual proteasome activity at C2D15 (Cycle 2 Day 15) post treatment compared to baseline proteasome activity levels in PBMCs obtained from patients #1 to #5 (shaded bars). **G)** Quantification of the relative contribution of the $\beta 2 + \beta 2i$ activities to total proteasome activity in patients #1 to #5 during treatment cycles 1 and 2 in PBMC samples in which activity has recovered (pre treatment samples). **H)** Quantification of the relative contribution of constitutive proteasome to total proteasome activity in patients #1 to #5 during treatment cycles 1 and 2 in PBMC samples in which activity has recovered (pre treatment samples).

#1 to #5 in which activity had recovered (pre treatment samples obtained during cycles 1 and 2). Both the contribution of the $\beta 2$ activity to total proteasome activity (defined as the sum of the $\beta 2$ and $\beta 2i$ activities, which are least targeted by marizomib; Figure 4G), and the contribution of constitutive activities to total activity (Figure 4H) were calculated. In patients #1 and #4, in which proteasome activity fully recovered between marizomib administrations, the relative $\beta 2$ activity increased steadily over time in response to marizomib treatment, independent of the initial active subunit composition. In patients #2 and #3 in which proteasome activity did not fully recover between doses, the relative $\beta 2$ activity increased less in response to marizomib treatment, while in patient #5 in which least proteasome activity recovery was observed the relative $\beta 2$ activity somewhat decreased over time. In addition, the relative constitutive activity increased in all patients with the exception of patient #3. A similar shift from immunoproteasome to constitutive proteasome was observed *in vitro* in cells with acquired resistance to bortezomib. In these cells, this shift was accompanied by the upregulation of constitutive activities that could no longer be inhibited by bortezomib and of apoptosis resistance.³⁰ Together, these data suggest that the rapid modification of proteasome composition and thereby cleavage

specificity allow PBMCs to adapt and survive marizomib treatment. The ability to upregulate $\beta 2$ activities, which are less sensitive to inhibition by marizomib, seems to correlate to the ability of PBMCs to fully recover proteasome activity between administrations. Whether upregulation of $\beta 2$ activities results in a decreased sensitivity and thereby less cell death in response to marizomib treatment remains to be determined. These data indicate that PBMCs modify their active subunit composition in response to marizomib treatment and that this may be a factor contributing to marizomib response.

DISCUSSION

Marizomib is a second-generation proteasome inhibitor isolated from the marine actinomycete *Salinispora tropica* that binds all active proteasome subunits irreversibly.⁹ Marizomib has been evaluated in a wide range of preclinical models and has demonstrated clinical activity against both hematologic and solid tumors.²¹ Clinical trials with marizomib are currently ongoing in both hematologic and solid tumors, enabling for the first time a thorough evaluation of patient response to marizomib treatment. Proteasome probe **1** is a recently developed reagent that enables profiling of all proteasome activities in a single measurement in a wide range of

tissue types, both *in vitro* and *ex vivo*.²⁷ The present study shows that probe **1** can be used to profile the effects of proteasome inhibitors on blood samples, derived both from animals and, importantly, from patients that are treated with this type of drug. Inhibition profiles of marizomib and bortezomib in rat PBMCs were in accord with previous reports^{9,26} and revealed that marizomib inhibited all subunits with the highest affinity for $\beta 5$ subunits, while bortezomib inhibited the $\beta 5$ and $\beta 1$ but not the $\beta 2$ subunits. Parallel measurement of the proteasomal activities using both probe **1** and fluorogenic substrates in patient PWB samples gave similar results, further validating the use of probe **1** for assaying proteasome activity in blood samples. It has been shown that measuring only the chymotryptic activity may not accurately reflect the total proteasome activity, and that both the caspase-like and tryptic activities may considerably contribute to overall proteasome activity.¹⁹ The use of chemical probes to assay proteasome activity has the advantage that the activity of all constitutive and immunoproteasome subunits can be profiled in a single measurement. In addition, probe **1** has equal affinity for all proteasome active sites and can therefore be used to establish the relative contribution of each subunit to total proteasome activity and to reliably measure total proteasome activity. Recovery patterns in PWB, which consists predominantly of erythrocytes (>99%) that are devoid of a nucleus, differed substantially from results found in PBMCs. Whereas in rat PBMCs total proteasome activity recovered within 72 h to 168 h post dosing, proteasome activity in PWB samples was only recovered by 20 to 50% after 168 h. In patients, PBMC proteasome activity recovered almost completely between doses, while recovery was near absent in PWB. These differences in recovery can be explained by the inability of erythrocytes to synthesize proteins, which makes

proteasome activity recovery completely dependent on the slow turnover of erythrocytes ($t_{1/2}$ = 15-17 weeks).²⁵ It has been suggested previously that blood proteasome inhibition does not correlate with tumor proteasome inhibition or inhibitor efficacy.^{11,29} The data presented here are a further indication that erythrocytes respond to and recover from proteasome inhibitors uniquely and caution against the use of PWB as a model system to monitor the effects of proteasome inhibitors in animals or patients, as is routinely done.

Probe **1** enabled a detailed study of patient response to marizomib treatment. Although proteasome activity was monitored in a small group of patients, factors that may mediate sensitivity to proteasome inhibition could be derived from these data. The data presented here indicate for the first time that marizomib treatment results in inhibition of similar absolute amounts of proteasome activity in both PWB and PBMCs. This suggests that both the relative and absolute residual proteasome activities after marizomib treatment are determined by the initial level of total proteasome activity. Therefore, total proteasome activity is likely one of the factors determining the sensitivity of cells to marizomib and possibly other proteasome inhibitors, as has been suggested earlier.^{8,31} In accord with this, cells with acquired bortezomib resistance have shown increased expression and activity of the proteasome *in vitro*.^{30,32,33} Initial proteasome activity in PWB seemed to correlate to initial activity in PBMCs, suggesting that proteasome activity levels may be regulated similarly in different cell types. Therefore, the initial proteasome activity in blood may be indicative of the initial activity in hematologic malignancies. Measuring the initial proteasome activity in PWB compared to a benchmark sample may thus be an easy approach to determine whether a patient is likely to respond to proteasome inhibitor therapy and could be a first

step towards personalized proteasome inhibitor therapy.

The ability of cells to shift their active subunit composition in response to proteasome inhibitor therapy may be another factor that mediates sensitivity to this class of compounds. Patient PBMCs responded to marizomib therapy by shifting their proteasome activity profile towards a higher degree of $\beta 2$ activity and a higher degree of activity of constitutive subunits, in line with observations made *in vitro*.³⁰ These shifts probably enable PBMCs to better survive proteasome inhibition. Therefore, the (partial) absence of these shifts in tumor cells likely renders cells more vulnerable to proteasome inhibition. It is tempting to speculate that certain tumor types lack the ability to shift their active subunit composition in response to proteasome inhibitor therapy, suggesting that tumor types that are especially sensitive to proteasome inhibitors could be identified. Alternatively, it would be interesting to define the cellular mechanisms that mediate these shifts, as pharmacological interference with such pathways may overcome resistance to and enhance the therapeutic potential of proteasome inhibitors.

Finally, differences in initial subunit activity distribution are likely to influence the response of tumors to different proteasome inhibitors, as was suggested previously by Krauss et al.³² Both red and white blood cells, which have a relatively high, micromolar intracellular proteasome concentration,²⁵ are thought to sequester proteasome inhibitors *in vivo*,²⁹ as blood cells are the first cell type to come in contact with intravenously administered proteasome inhibitors. Consequently, proteasome inhibitors likely saturate proteasomes in red blood cells, which constitute 99% of all blood cells, before peripheral sites can be reached,²⁹ which may limit inhibitor concentrations in tumor cells. PR-957 is a novel $\beta 5i$ -specific proteasome inhibitor that showed ac-

tivity against immunoproteasomes in mice.³⁴ As red blood cells do not express immunoproteasome, specific immunoproteasome inhibitors may saturate blood proteasome at relatively low concentrations, resulting in higher inhibitor concentrations in tumor cells. In addition, immunoproteasome inhibitors are suggested to cause fewer side effects in tissues that express only low levels of immunoproteasome subunits.⁸ Therefore, PR-957 or other immunoproteasome inhibitors may in future provide a good alternative for tumors that originate from immune tissues that have intrinsic immunoproteasome activity, such as spleen, lymph nodes and thymus.²⁷ The data presented here also suggest that the relative contribution of subunits to total proteasome activity may differ between patients and similar observations were made in primary human leukemia cells.³² In addition, analysis of rat PBMCs suggest that proteasome inhibitor profiles may differ substantially, especially at low concentrations. Whereas the $\beta 1i$ subunit was especially susceptible to bortezomib inhibition, the $\beta 2$ subunits could only be inhibited by marizomib. Probing tumor cells with probe **1** before treatment will establish which subunits are most active. This will enable matching of tumor proteasome profile to a complementary inhibitor or multiple inhibitors to personalize proteasome inhibitor therapy and maximize therapy response.

MATERIALS AND METHODS

Reagents

All solvents were purchased from Biosolve (Valkenswaard, the Netherlands) at the highest grade available. All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) at the highest available purity. Bortezomib (obtained commercially) and marizomib synthesized at Nereus (San Diego, CA). Probe **1** was synthesized as described in chapter 2.1.

Ex vivo profiling rat blood samples using fluores-

cent probe 1

Male Sprague-Dawley rats (280-325 grams) were treated once intravenously with 40% Propylene glycol/50% citrate buffer/10% ethanol based vehicle with or without marizomib (0.05 or 0.1 mg/kg) or bortezomib (0.15 mg/kg in saline). Fifteen rats served as untreated controls. At 90 minutes, 24, 48, 72, and 168 hours post dose, 3 rats per time point per dosing group were anesthetized and approximately 6-7 mL of whole blood was collected via the abdominal aorta into Na-Heparin vacutainer tubes. To obtain a PWB pellet, 2 mL of whole blood was centrifuged for 10 minutes at 800g; the blood pellet was then washed once with cold 1X PBS (supernatant was discarded) and frozen at -80 °C. PBMCs were isolated using Lympholyte®-Mammal (Cedarlane Laboratories, Ontario, Canada): 4 mL of whole blood was diluted 1:1 with 4 mL of PBS and the tube was inverted 2-3 times to mix; 4 mL of the diluted blood was then layered onto 3 mL of Lympholyte®-Mammal in a 15 ml centrifuge tube and centrifuged at room temperature for 20 minutes at 800g. The PBMC layer at the interface was transferred to a new centrifuge tube, diluted 1:1 with PBS, and pelleted for 10 minutes at 800g. The supernatant was discarded; the PBMCs were washed once in PBS and frozen at -80 °C.

PBMC and PWB pellets were thawed on ice. 50 µL of ice-cold 5 mM EDTA pH8 lysis buffer was added to the PBMC pellets, and 2 mL lysis buffer to the PWB pellets. Pellets were resuspended and lysed on ice for 1 h. Lysates were then centrifuged for 10 min at 16,000 x g at 4 °C, and the supernatants were aliquoted and stored at -80°C. Protein concentration was determined using a Bradford assay (Biorad). Protein concentrations were adjusted to 2 µg/µL with HR-buffer (50 mM Tris-HCl pH 7.4, 250 mM sucrose, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 2 mM ATP), and lysates were incubated with 1 µM probe 1 for 1 hour at 37 °C. Data were analyzed by using GraphPad Prism software (GraphPad, La Jolla, CA, USA).

Ex vivo profiling patient blood samples

At indicated time points according the clinical protocol, 5 mL of blood was drawn in heparinized vacutainers. Blood samples were obtained pre dosing or within 2.5 h to 5 h post dosing for all patients. 1 mL of the heparinized whole blood was centrifuged at 2000g for 10 min at room temper-

ature (RT) and the PWB pellet was washed once in 5 times the pellet volume of ice-cold 1X DPBS (Dulbecco's Phosphate Buffered Saline) with Ca²⁺/Mg²⁺. After centrifugation (2000g, 10 min RT) and discarding of supernatants, PWB pellets were frozen and stored at -70°C. The remaining 4 mL of heparinized whole blood was diluted by adding an equal volume of Hanks Balanced Salt Solution and layered over 6 mL of Ficoll-Paque™ PLUS. After centrifugation for 40 min at 400g at room temperature (RT), PBMCs located at the interphase were collected, transferred to a new tube, and washed with at least three volumes of Hanks Balanced Salt Solution. To lyse any co-isolated RBCs, PBMC pellets were resuspended in 1x BD PharmLyse™ buffer and incubated at RT for 5 minutes. 9 mL of Hanks Balanced Salt Solution was then added and cells were centrifuged for 10 min at 1000g (RT). PBMCs were resuspended in Hanks Balanced Salt Solution, an aliquot was taken for a viability cell count by a Flow based 7-Amino-actinomycin D (7-AAD) staining procedure and the remaining PBMC solution was centrifuged for 10min at 1000g (RT). Supernatant was discarded, and PBMC cell pellets were flash frozen and stored at -70 °C.

For *ex vivo* profiling of proteasome activities using probe 1, cell pellets were lysed in HR lysis buffer (50 mM Tris pH 7.4, 1 mM dithiothreitol (DTT), 5 mM MgCl₂, 2 mM ATP and 250 mM sucrose) by sonification using the Bioruptor sonicator (Diagenode, Liège, Belgium). Lysates were centrifuged at 16,000g to remove cell debris and protein concentration was determined using the Bradford assay (Biorad). Protein concentrations were adjusted to 1 µg/µL (PBMC) or 5 µg/µL (PWB), and lysates were incubated with 1 µM probe 1 for 1 h at 37 °C. To measure proteasome activities *ex vivo* using fluorogenic substrates, cells were lysed in ice-cold 5 mM EDTA pH8 lysis buffer and protein concentrations were determined using a BCA (bicinchoninic acid) assay. The 20S proteasome activities in cell lysates were measured as described previously.^{25,35} In brief 20 µg of protein was used in a total volume of 200µL consisting of assay buffer (20 mM HEPES, 0.5 mM EDTA, pH 8.0) with a final SDS concentration of 0.035% and 20 µM of the peptide substrates Suc-LLVY-AMC (Chymotryptic activity), Z-ARR-AMC (Tryptic activity) or Z-LLE-AMC (caspase-like activity) (Calbiochem). Fluorescence (λ ex/em = 390/460 nm)

was measured every 5 min for 2 hr using a Fluoroskan Ascent 96-well microplate reader (Thermo Electron, Waltham, MA).

In-gel fluorescence measurements

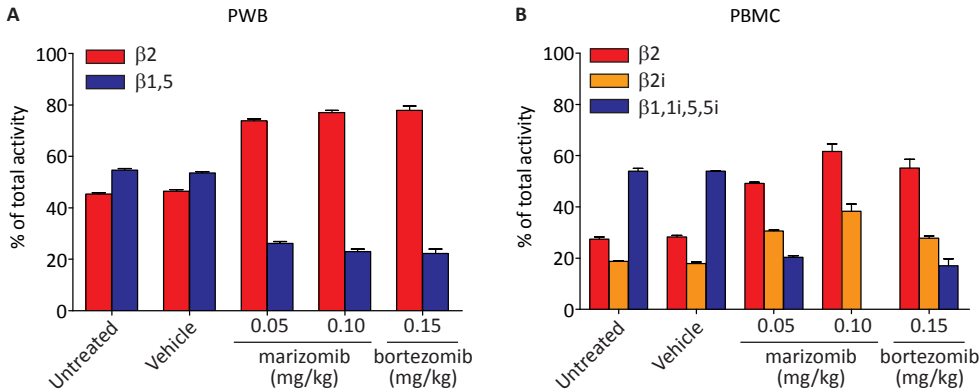
Incubated samples were denatured by boiling in LDS sample buffer (Invitrogen) containing 2.5% β -mercaptoethanol and polypeptides were resolved by 12% Bis-Tris gels using the NuPAGE system from invitrogen. Wet gel slabs were imaged for 10s to 120s, with a resolution of 100 μ m, using the ProXPRESS 2D Proteomic imaging system (Perkin Elmer) with appropriate filter settings ($\lambda_{ex/em}$ = 480/530 nm). To verify protein loading, gels were stained with coomassie and probe signals were normalized accordingly. Images were analyzed using TotalLab analysis software (Non-linear Dynamics, Newcastle upon Tyne, UK) to quantify the intensity of the bands detected. To compare baseline proteasome activities between patients, samples were rerun on a single gel.

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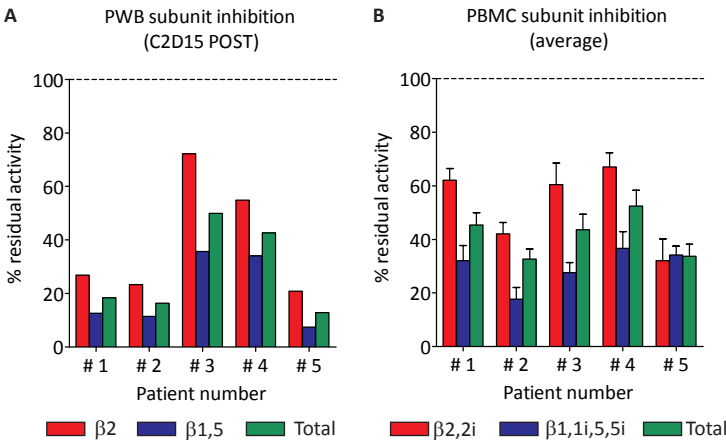
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Supplementary figures



Supplementary Figure 1 | Proteasome activity profiles are identical in untreated and vehicle treated rat blood samples. A) Quantification of the relative contribution of the $\beta 2$ and $\beta 5/1$ subunits to total proteasome activity in rat PWB samples 1.5 h post treatment. **B)** Quantification of the relative contribution of the $\beta 2$, $\beta 2i$ and $\beta 5/5i/1/1i$ subunits to total proteasome activity in rat PBMC samples 1.5 h post treatment.



Supplementary Figure 2 | Residual proteasome activities in marizomib treated patients. A) Quantification of the residual activity of individual subunits after marizomib treatment in patient PWB samples normalized to baseline activity levels. **B)** Quantification of the residual activity of individual subunits after marizomib treatment in patient PBMC samples. Activities in post treatment samples were normalized to the preceding pre treatment sample and averaged over all administrations. Error bars represent SEM.

