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Clinical activity of carfilzomib correlates with inhibition of multiple proteasome subunits: application of a novel pharmacodynamic assay

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Summary

While proteasome inhibition is a validated therapeutic approach for multiple myeloma (MM), inhibition of individual constitutive proteasome (c20S) and immunoproteasome (i20S) subunits has not been fully explored owing to a lack of effective tools. We utilized the novel proteasome constitutive/immunoproteasome subunit enzyme-linked immunosorbent (ProCISE) assay to quantify proteasome subunit occupancy in samples from five phase I/II and II trials before and after treatment with the proteasome inhibitor carfilzomib. Following the first carfilzomib dose (15–56 mg/m²), dose-dependent inhibition of c20S and i20S chymotrypsin-like active sites was observed [whole blood: ≥67%; peripheral blood mononuclear cells (PBMCs): ≥75%]. A similar inhibition profile was observed in bone marrow-derived CD138⁺ tumour cells. Carfilzomib-induced proteasome inhibition was durable, with minimal recovery in PBMCs after 24 h but near-complete recovery between cycles. Importantly, the ProCISE assay can be used to quantify occupancy of individual c20S and i20S subunits. We observed a relationship between MM patient response ($n = 29$), carfilzomib dose and occupancy of multiple i20S subunits, where greater occupancy was associated with an increased likelihood of achieving a clinical response at higher doses. ProCISE represents a new tool for measuring proteasome inhibitor activity in clinical trials and relating drug action to patient outcomes.

Keywords: molecular analysis, multiple myeloma, myeloma therapy, pharmacology, trials.

The 26S proteasome is a ubiquitously expressed protein complex responsible for the homeostatic control of protein turnover and regulated degradation of proteins involved in most cellular functions (Wilk & Orłowski, 1983; Coux *et al*, 1996; Ciechanover, 2012). Each proteasome has a 20S core, each containing two copies of three distinct proteolytic enzymes. The proteasome exists in two forms: the constitutive proteasome (c20S), expressed ubiquitously throughout the body, and the immunoproteasome (i20S), expressed primarily in haematopoietic cells or in cytokine-exposed non-haematopoietic cells (Martinez & Monaco 1991; Glynn *et al*, 1991, Nandi *et al*, 1996). In the c20S, proteolytic activities are encoded in the β 5, β 1 and β 2 subunits and are characterized on the basis of substrate specificity as chymotrypsin-like (CT-L), caspase-like and trypsin-like, respectively. In the i20S, low-molecular mass polypeptide 7 (LMP7), LMP2 and multicatalytic endopeptidase complex-like 1 (MECL1) replace β 5, β 1 and β 2, respectively.

Approval of the dipeptide boronic acid proteasome inhibitor, bortezomib, for the treatment of relapsed multiple myeloma (MM; Richardson *et al*, 2005) and relapsed or refractory mantle cell lymphoma (Fisher *et al*, 2006) has spurred the development of other agents that target proteasome activity (Bennett & Kirk, 2008; Dick & Fleming, 2010; Kuhn *et al*, 2011). These agents vary from targeting individual active sites to targeting all three catalytic activities (Chauhan *et al*, 2005; Muchamuel *et al*, 2009). Carfilzomib, a tetrapeptide epoxyketone proteasome inhibitor, has recently been approved in the US for the treatment of relapsed and refractory MM (Kuhn *et al*, 2007; Jain *et al*, 2011). While both carfilzomib and bortezomib primarily target the CT-L activity of the c20S and i20S, carfilzomib displays greater specificity than bortezomib for CT-L activity relative to caspase-like and trypsin-like activities (Demo *et al*, 2007).

Accurate and relevant markers to monitor pharmacodynamics are important in the development of targeted agents for cancer, in which narrow therapeutic margins may limit drug effectiveness (Kummar *et al*, 2010; Yap *et al*, 2010). Ideally, direct measurement of target inhibition in malignant cells should be related to patient outcome. This approach is challenging given the complexity of the target (particularly multi-subunit intracellular protein complexes), the need for serial sampling, and the current lack of ready access to tumour cells. Instead, surrogate tissues (e.g. cells isolated from blood) and/or markers (e.g. changes in downstream signalling pathways) are used to approximate the clinical profile of an agent. This method may result in either equivocal results or the incorporation of potentially suboptimal dosing strategies into later clinical trials.

The standard method for determining proteasome inhibition uses biochemical substrates, such as Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (LLVY-AMC) for determining CT-L activity (Lightcap *et al*, 2000); however, this approach cannot differentiate between c20S and i20S activity. MM cells express both proteasome types, and the role of individual

subunits in the anti-tumour effect of clinically relevant proteasome inhibitors remains an area of active research (Kuhn *et al*, 2009; Parlati *et al*, 2009; Wehenkel *et al*, 2012). Meanwhile, the role of the non-CT-L active sites and their inhibition by proteasome inhibitors remain poorly understood, mainly due to a lack of suitable substrates for measuring enzymatic activity. Substrates for LMP2 and MECL1 are poorly defined and, similar to LLVY-AMC, do not differentiate between immunoproteasome subunit activity and the corresponding constitutive proteasome subunits (Groettrup *et al*, 2010). Studies utilizing inhibitors that specifically target the caspase-like or trypsin-like active sites suggest that inhibition of non-CT-L active sites alone may sensitize cancer cells to CT-L inhibition but are not sufficient to induce cytotoxicity (Britton *et al*, 2009; Mirabella *et al*, 2011). Other assessment tools are needed to fully characterize the role of c20S and i20S active sites in the anti-cancer activity of proteasome inhibitors. Considering these limitations, we developed the proteasome constitutive/immunoproteasome subunit enzyme-linked immunosorbent (ProCISE) assay to measure the amounts and occupancy of proteasome active sites in samples with heterogeneous proteasome composition (Parlati *et al*, 2009). We previously reported the use of the ProCISE assay in quantifying c20S and i20S subunit levels in primary cells and cell lines derived from haematopoietic and solid tumours (ST) (Parlati *et al*, 2009). Herein, we present the first clinical application of ProCISE, demonstrating its utility in determining the level of occupancy of all six proteasome active sites in samples derived from MM and ST patients treated with carfilzomib from five phase I/II and phase II clinical trials.

Methods

Reagents

Proteasome active site-binding probe (PABP) (Bennett *et al*, 2006) and carfilzomib (Smyth & Laidig, 2006) were synthesized as previously described (Smyth & Laidig, 2006). The fluorogenic substrate succinyl-LLVY-AMC and human c20S and i20S were purchased from Boston Biochem (Boston, MA, USA). Human whole blood and peripheral blood mononuclear cell (PBMC)-enriched leukapheresis-derived samples from healthy volunteers were purchased from All-Cells (Berkeley, CA, USA). Monoclonal antibodies specific for β 1, β 2, LMP7 and LMP2 were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Antibodies specific to MECL1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A rabbit polyclonal anti- β 5-antibody was generated as previously described (Muchamuel *et al*, 2009). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse were purchased from Jackson ImmunoResearch (West Grove, PA, USA). HRP-conjugated rabbit anti-goat was purchased from Invitrogen (Carlsbad, CA, USA).

Table I. Clinical Samples used for Pharmacodynamic Analysis.

Blood and PBMC pharmacodynamic samples from MM patients*				
Trial†	Phase	N	2- to 10-min infusion dose (initial/escalation mg/m ²)	30-min infusion dose (initial/escalation mg/m ²)
PX-171-005 (NCT00721734)	II	31	15/20 (N = 31)	—
PX-171-006 (NCT00603447)	Ib/II	16	15 only (n = 8) 20/27 (n = 8)	—
PX-171-007 (NCT00531284)	I/II	24		20/36 (n = 3) 20/45 (n = 3) 20/56 (n = 19)
Bone marrow pharmacodynamic samples from MM patients‡				
Trial†	Phase	N	2- to 10-min infusion dose (mg/m ²)	30-min infusion dose (mg/m ²)
PX-171-003-A0 and -A1 (NCT00511238)	II	7	20 only (N = 7; predose, n = 3; C1D2, n = 4)	—
PX-171-004 (NCT00530816)	II	11	20 only (N = 11; predose, n = 6; C1D2, n = 5)	—
PX-171-005 (NCT00721734)	II	15	Dose N/A (MM, N = 15; predose, n = 15; C1D2, n = 0)	—
Blood and PBMC pharmacodynamic samples from ST patients*				
PX-171-007 (NCT00531284)	I/II	36	20 only (n = 3) 20/27 (n = 3) 20/36 (n = 18)	20/45 (n = 2) 36 only (n = 6) 45 only (n = 6)

C, cycle; D, day; MM, multiple myeloma; N/A, not applicable; PBMC, peripheral blood mononuclear cell; ST, solid tumour.

*cycle 1, multiple days, and cycle 2, day 1 only.

†Blood and/or bone marrow aspirates were collected from patients in five clinical trials prior to and after carfilzomib treatment and at the doses listed. Samples were analysed for proteasome activity and subunit occupancy as described in Fig 1.

‡Predose and C1, D2 only.

Sample collection

Samples for analysis were derived from phase I/II and phase II trials (Table I). Trial details can be found in the Supplementary Information. Patients received carfilzomib as an intravenous infusion over 2–10 min or, as in the case of PX-171-007, over 30 min on days 1, 2, 8, 9, 15 and 16 of a 28-day cycle at doses from 15 to 56 mg/m².

In PX-171-003-A0/A1, PX-171-004 and PX-171-005, bone marrow samples were collected during screening and on day 2 of cycle 1 (approximately 4 h post-dose). In PX-171-005, PX-171-006 and PX-171-007, whole blood and isolated PBMC samples were collected prior to dosing and 1 h post-dose on days 1, 2 and 8 of cycle 1 and on day 1 of cycle 2 (no samples were collected on days 2 or 8 in cycle 1 for patients with ST receiving carfilzomib over 2–10 min). All samples were obtained after informed consent was given and in accordance with the Helsinki Declaration.

Sample preparation

Bone marrow aspirates were enriched for CD138⁺ tumour cells via immunomagnetic bead separation (RoboSep, Stem Cell Technologies, Vancouver, Canada) as per the manufacturer's protocol. Cells were lysed in lysis buffer (20 mM Tris pH 8.0, 5 mM ethylenediaminetetraacetic acid, EDTA) and cleared of cellular debris by centrifugation prior to analysis.

CT-L activity assay

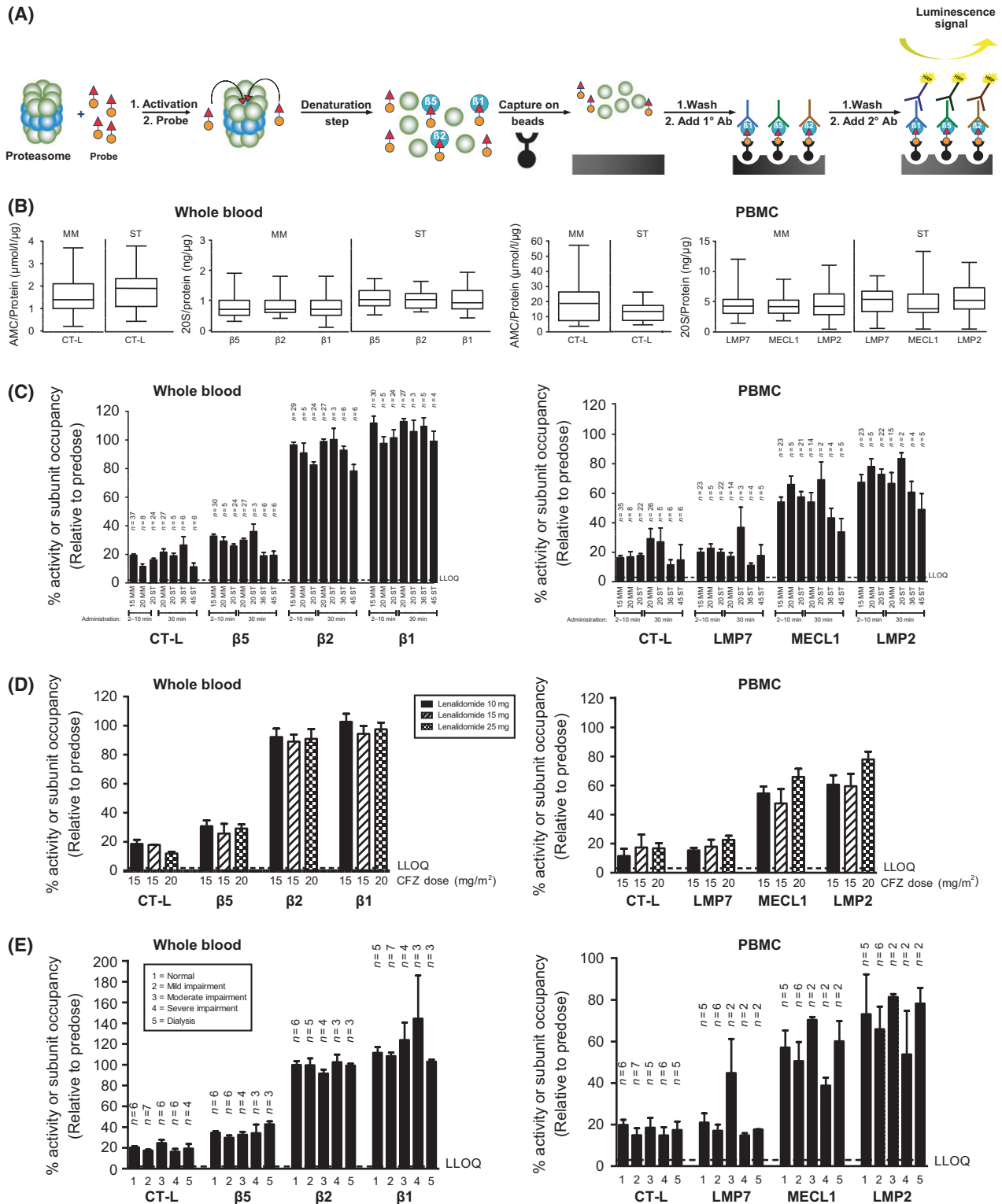
CT-L activity using succinyl-LLVY-AMC was determined as previously described (Lightcap *et al*, 2000; Demo *et al*, 2007). Protocol modifications for the current study included a blood quality control (QC) matrix, generation of an aminomethylcoumarin standard curve and QC samples. Details are provided in the Supplementary Information.

ProCISE assay

We previously published the methodology of the ProCISE assay for evaluation of samples from primary cells and tumour cell lines (Parlati *et al*, 2009). The clinical application of the ProCISE assay involves, in brief, the following steps: (i) incubation of PABP with whole blood, PBMC or bone marrow lysates; (ii) denaturation with 5.6 M guanidine hydrochloride; (iii) addition of streptavidin-coated beads; (iv) extensive bead washing; (v) addition of proteasome subunit-specific primary antibody followed by a secondary HRP-conjugated antibody; and (vi) luminescence-based detection. Additional details on the clinical application of the ProCISE assay can be found in the Supplementary Information section.

Statistical analysis

One-tailed Welch–Satterthwaite *t*-tests, or fixed, or mixed-effects analysis of variance models followed by Tukey's



studentized range *post-hoc* tests were used, as appropriate, to determine statistically significant differences. The presence of a linear trend was tested using an F-test for linear contrast. Statistical analyses were performed using GraphPad Prism

(version 5.03; GraphPad Prism Software Inc., San Diego, CA, USA) or SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC, USA). Statistical significance was achieved when $P < 0.05$.

Fig 1. Carfilzomib administration results in potent inhibition of multiple proteasome subunits. (A) ProCISE assay schematic. PABP is added to purified 20S proteasomes (activated with 0.03% SDS-AB) or cell lysates and incubated for 2 h. Denaturation with guanidine and capture on beads is then done simultaneously for 1 h followed by five washes in PBS + 1% BSA + 0.05% Tween. Primary antibodies for specific proteasome active site subunits are then added overnight, followed by another five washes. A secondary HRP antibody is then added for 2 h, followed by five washes. A chemiluminescent substrate is then added for 5–10 min, and luminescence is determined. (B) Proteasome subunit levels were measured by ProCISE in 62 whole blood and 54 PBMC samples from MM patients and 27 whole blood and 24 PBMC samples from ST patients. CT-L activity was measured in the same samples using the LLVY-AMC substrate. Data are presented as box and whisker plots with subunit concentration (ProCISE) or specific activity (LLVY-AMC). Mean specific activity [AMC/protein ($\mu\text{M}/\mu\text{g}$)] and subunit concentrations [subunit/protein ($\text{ng}/\mu\text{g}$)] are represented by a horizontal line, and error bars represent minimum and maximum values. (C) Whole blood and PBMC samples taken 1 h after the first administration of carfilzomib in MM and ST patients (doses: 15–45 mg/m^2) were analysed for proteasome subunit activity by LLVY-AMC and occupancy by ProCISE. Values were normalized to predose values, and data are presented as mean ($\pm\text{SEM}$) relative activity or occupancy. The dotted line represents the LLOQ for whole blood. (D) MM patients were treated with carfilzomib 15 or 20 mg/m^2 , dexamethasone 40 mg and lenalidomide 10, 15, or 25 mg on day 1. Whole blood and PBMC were isolated predose and 1 h after the first dose of carfilzomib dose was administered. Samples were analysed for proteasome activity and subunit occupancy, as described in panel A. Values are normalized to predose values, and data are presented as mean ($\pm\text{SEM}$) specific activity or occupancy ($N = 2\text{--}8$ per cohort). LLVY-AMC data are reproduced from Badros *et al* (2013) with permission from Nature Publishing Group. (E) MM patients with varying degrees of renal function were treated with 15 mg/m^2 of carfilzomib on day 1. Whole blood and PBMC were isolated predose 1 h after carfilzomib dose administration. Samples were analysed for proteasome activity and subunit occupancy as described in panel B. Values are normalized to predose values, and data are presented as mean ($\pm\text{SEM}$) relative activity or occupancy ($N = 2\text{--}7$ per cohort). 1°, primary; 2°, secondary; Ab, antibody; AMC, aminomethylcoumarin; BSA, bovine serum albumin; CFZ, carfilzomib; CT-L, chymotrypsin-like; D, day; HRP, horseradish peroxidase; LLOQ, lower limit of quantitation; LLVY, Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; LMP, low-molecular mass polypeptide; MECL, multicatalytic endopeptidase complex-like; MM, multiple myeloma; PABP, proteasome active site-binding probe; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffer solution; SDS-AB, sodium dodecyl sulfate in assay buffer; SEM, standard error of the mean; ST, solid tumour.

Results

ProCISE clinical validation

ProCISE quantitatively measures the levels of proteasome subunit active sites within cell lysates via the addition of a biotinylated probe that covalently binds to all active sites. Probe-bound active sites are captured on streptavidin-sepharose beads and quantified with subunit-specific antibodies compared with a standard curve of purified proteasomes (Fig 1A). We determined that ProCISE accurately measures c20S and i20S subunits within a large dynamic range (2–167% of c20S subunits and 3–250% of i20S subunits found within typical whole blood and PBMC samples, respectively; Table SI). In addition, when known concentrations of c20S and i20S were spiked into whole blood or PBMC samples, respectively, we observed good correlation between the measured and expected values (Figure S1).

To validate ProCISE as a measure of drug activity, we treated whole blood for 1 h with varying concentrations of carfilzomib and analysed both whole blood and isolated PBMCs (Figure S2). The *ex vivo* dose response of carfilzomib-mediated $\beta 5$ and LMP7 occupancy measured by ProCISE mirrored that of the enzymatic activity assessed using LLVY-AMC in the same samples. Carfilzomib showed more than 10-fold selectivity for CT-L subunits relative to other c20S and i20S subunits, findings that were similar to previously reported results from purified proteasomes (Demo *et al*, 2007). Finally, baseline levels of proteasome active sites from MM and ST patient samples collected from multiple studies were analysed by ProCISE (Fig 1B). Stoichiometric amounts of the c20S subunits and the i20S subunits were present in whole blood and PBMCs, respectively,

independent of tumour type. Additionally, levels of CT-L activity inhibition and subunit occupancy in patients receiving carfilzomib were similar using the LLVY-AMC or ProCISE assays in whole blood or PBMCs, suggesting that measurement of subunit occupancy accurately reflects proteolytic activity (Fig 1C; Table SII). Taken together, these data validate ProCISE for the quantitative measurement of proteasome inhibition, active site occupancy and levels of active site subunits in patient samples.

Inhibition of proteasome subunits by carfilzomib in patients

Carfilzomib has demonstrated anti-tumour activity in patients with MM at doses ranging from 15 to 56 mg/m^2 (Siegel *et al*, 2012; Vij *et al*, 2012a,b; Badros *et al*, 2013; Papadopoulos *et al*, 2013, 2015). In phase I and II trials, only the LLVY-AMC assay was used in a limited number of patient samples to measure CT-L activity alone (O'Connor *et al*, 2009; Badros *et al*, 2013; Niesvizky *et al*, 2013; Papadopoulos *et al*, 2013). To obtain the full active-site inhibition profile of carfilzomib, we utilized ProCISE to quantify active site activity and occupancy in whole blood and PBMC samples from patients in multiple studies ($n = 41$ ST and $n = 73$ MM) 1 h after the first dose of carfilzomib (15–45 mg/m^2) was administered. In patients receiving 15 or 20 mg/m^2 of carfilzomib as a 2- to 10-min infusion, rates of inhibition of $\beta 5$ and LMP7 occupancy in whole blood and PBMC samples were 67–74% and 77–80%, respectively (see Fig 1C). At 20 mg/m^2 , no significant difference in the level of proteasome inhibition was detected between patients receiving a 2- to 10-min infusion and those receiving a 30-min infusion. Inhibition of the other i20S subunits (MECL1 and LMP2) was also observed in PBMCs at doses of 15 and

20 mg/m², albeit at lower levels than LMP7 inhibition (31–46% inhibition of MECL1 and 22–33% inhibition of LMP2). At the same doses, there was less than 18% inhibition of the c20S subunits β 2 and β 1 in whole blood samples. At a higher dose (45 mg/m² administered as a 30-min infusion), we noted increased occupancy for almost all subunit sites assessed (Fig 1C). The effect was most prominent for MECL1 and LMP2; occupancy of β 1, however, was not observed. A statistically significant linear dose-response effect was seen for occupancy of β 5, β 2 and MECL1 (*P* values were 0.0044, 0.0137 and 0.0432 for the linear trend, respectively).

Carfilzomib has been used in combination with lenalidomide and dexamethasone and as a single agent in patients with relapsed and/or refractory MM and varying degrees of renal function (Badros *et al*, 2013; Niesvizky *et al*, 2013; Siegel *et al*, 2013). Whole blood and PBMC samples from patients receiving 15 or 20 mg/m² of carfilzomib with 40 mg of dexamethasone and 10, 15 or 25 mg of lenalidomide (study PX-171-006) were taken 1 h after the first dose of carfilzomib and analysed for active site activity and occupancy. Levels of active site occupancy were similar in patients receiving carfilzomib in combination with varying doses of lenalidomide (Fig 1D). No effect from the co-administration of lenalidomide was noted at later time points in cycle 1 or at the beginning of cycle 2 (data not shown). In addition, active site occupancy was similar when patients treated with 15 mg/m² of carfilzomib were stratified based on renal function status (study PX-171-005) and was consistent with results previously reported using the LLVY-AMC assay (Fig 1E) (Badros *et al*, 2013). Taken together, these data demonstrate that carfilzomib induces consistent and potent occupancy of multiple proteasomal active sites in MM patients, regardless of concomitant drug administration or renal function.

Sustained proteasome inhibition after repeated carfilzomib administration

As carfilzomib selectively and irreversibly binds to its target (Demo *et al*, 2007), we assessed the duration of subunit occupancy and recovery of proteasome activity following its use. Whole blood and PBMC samples were analysed from patients from multiple studies both prior to and 1 h after receiving carfilzomib on days 1, 2 and 8 of cycle 1 and on day 1 of cycle 2. Consistent with results from animal studies (Demo *et al*, 2007), we observed cumulative and sustained subunit occupancy in whole blood samples that was most evident for β 2 and β 5 (Fig 2A). In PBMCs, modest recovery was seen 24 h after the first dose (day 2, predose), but unbound subunit levels were lower than pretreatment values (see Fig 2A). Following the second dose of carfilzomib, a slight increase in occupancy was observed across all i20S subunits (day 2, 1 h). Recovery following a 5-day non-dosing period (day 8, predose) was more prominent than after

a 24-h period (day 2, predose), but full recovery was not achieved. By the start of cycle 2 (cycle 2, predose), complete or near-complete recovery was noted in PBMCs for all three i20S active sites at doses below 36 mg/m². These results, in which data for each dose level were pooled across studies, are similar to those previously reported using the LLVY-AMC assay in patients treated with carfilzomib, lenalidomide and dexamethasone from PX-171-006 (Niesvizky *et al*, 2013). Our findings suggest that the addition of lenalidomide and dexamethasone to carfilzomib treatment does not impact the pharmacodynamics of carfilzomib-mediated proteasome inhibition. In patients receiving 45 mg/m² of carfilzomib, minimal recovery was observed between day 8 and the start of cycle 2; it should be noted, however, that relatively few patients (range, 3–9) were analysed at this dose level.

In study PX-171-007, patients were dose-escalated from 20 mg/m² to a target dose that ranged from 27 to 56 mg/m² beginning on day 8 of cycle 1. As near complete recovery of proteasome activity and subunit levels in PBMC was observed by the beginning of cycle 2 (cycle 2, predose), we compared the level of individual i20S subunit occupancy on cycle 2, day 1 with pretreatment values. Results from ProCISE showed that all doses resulted in similar and potent increases in LMP7 occupancy, regardless of whether carfilzomib was administered as a 2- to 10-min infusion or as a 30-min infusion, while a dose-dependent increase in MECL1 and LMP2 occupancy was observed at doses of up to 45 mg/m² (Figure S3). At a dose of 56 mg/m² (the maximum tolerated dose in patients with MM when infused over 30 min), carfilzomib produces near-complete LMP7 inhibition (i.e. at the limit of quantitation of the assay) and was bound to, on average, more than 79% of all i20S active sites.

We next determined whether the levels of proteasome inhibition detected in whole blood and PBMC samples were representative of target inhibition in tumour cells. As an optional correlative study, CD138⁺ tumour cells were isolated from bone marrow aspirates taken during screening and on cycle 1, day 2, and levels of β 5, LMP7 and MECL1 were assessed. Following administration of carfilzomib at a dose of 15 or 20 mg/m², we observed a statistically significant (>95%) reduction in CT-L activity as assessed by LLVY-AMC (*P* < 0.001). More moderate but still statistically significant reductions were seen at the β 5, LMP7 and MECL1 active sites [reductions of 52% (*P* < 0.05), 58% (*P* < 0.01) and 50% (*P* < 0.05), respectively] (Fig 2B). Intriguingly, we also observed that the i20S accounts for nearly 75% of the proteasome activity in these MM tumour cells (Table II). Not all samples could be tested in both assays because of the limited sample volume, which may account for discrepancies in the inhibition levels measured by LLVY-AMC and by ProCISE. Despite the limitations of this analysis, these data suggest that the use of carfilzomib results in inhibition lasting more than 48 h and that pro-

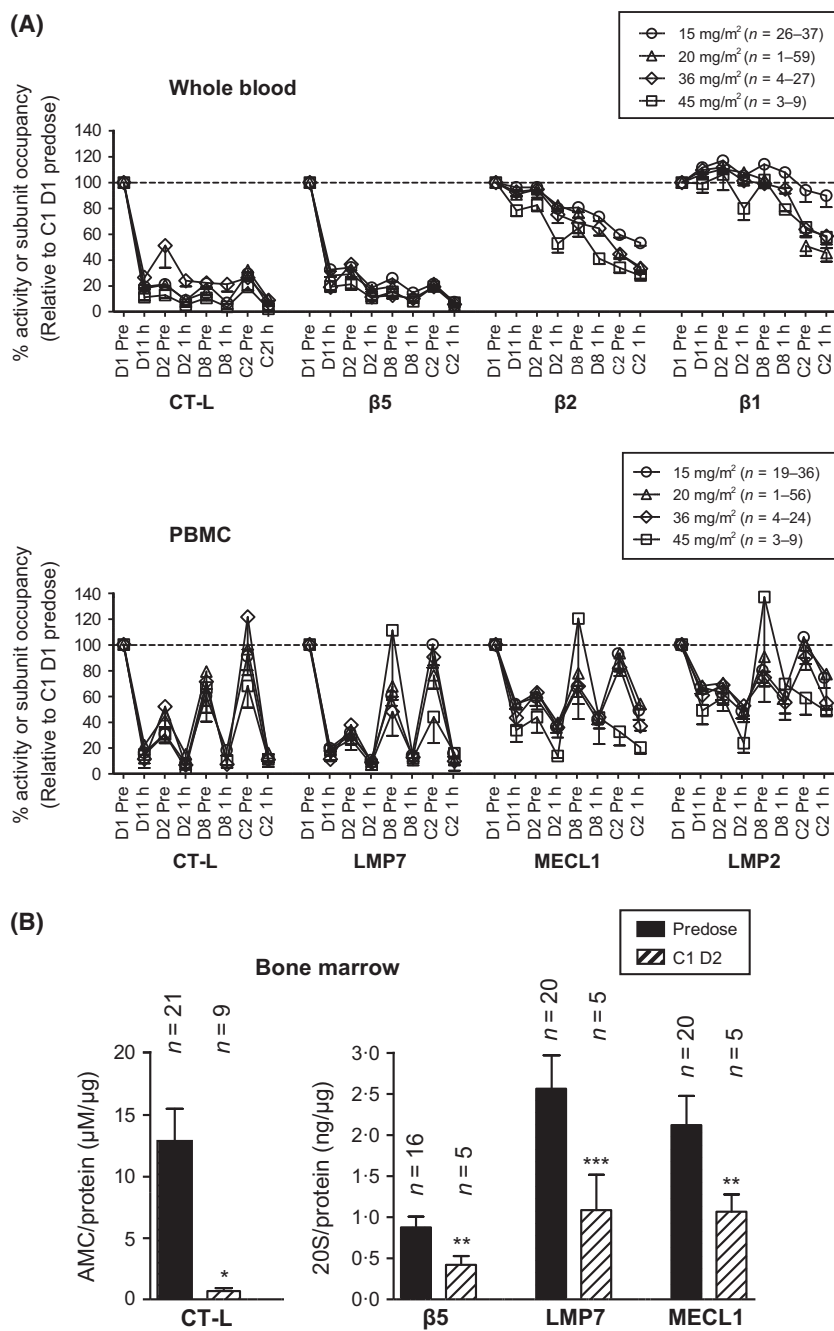


Fig 2. Repeat administration of carfilzomib results in sustained proteasome inhibition. (A) Patients received carfilzomib at 15, 20, 36 or 45 mg/m² on days 1, 2, 8, D9, 15 and 16 of a 28-day cycle. Whole blood and PBMC samples were taken prior to and 1 h after administration on days 1, 2 and 8 of cycle 1 and day 1 of cycle 2. Samples were analysed for proteasome activity and subunit occupancy as described in Fig 1 and were normalized to cycle 1, day 1 predose values. Data are presented as mean (±SEM) relative specific activity or occupancy. (B) Bone marrow samples from MM patients were taken during baseline screening and/or on cycle 1, day 2, 1 to 4 h after the second dose of carfilzomib. CD138⁺ cells and samples were analysed for proteasome activity and subunit occupancy. Data are presented as mean (±SEM) relative activity or subunit concentration. **P* < 0.001; ***P* < 0.01; ****P* < 0.05 by a *t*-test. C, cycle; CT-L, chymotrypsin-like; D, day; AMC, aminomethylcoumarin; LMP, low-molecular mass polypeptide; MECL1, multicatalytic endopeptidase complex-like; MM, multiple myeloma; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

Table II. CT-L Activity and Proteasome Subunit Levels in Baseline Primary Myeloma Cells and Multiple Myeloma Cell Lines.

Baseline CD138 ⁺ bone marrow proteasome activity and subunit composition					
Trials*	Average number of prior regimens (range)	CT-L (AMC/protein [μM/μg])	β5 (ng/μg protein)	LMP7 (ng/μg protein)	LMP7 (%)
PX-171-003, PX-171-004, PX-171-005	5 (1–13)	11.3 ± 2.8	0.9 ± 0.1	2.6 ± 0.4	74.3
Number of samples		29	18	22	

AMC, 7-amino-4-methylcoumarin; CT-L, chymotrypsin-like; LMP, low-molecular mass protein.

*Bone marrow aspirates were collected from patients in trials PX-171-003, PX-171-004 and PX-171-005 prior to carfilzomib dosing and sorted into CD138⁺ and CD138⁻ populations. CD138⁺ samples were analysed for proteasome activity and subunit occupancy as described in Fig. 1.

teasome inhibition in tumour cells is similar to that observed in PBMC samples.

Correlation between proteasome inhibition and response

To determine whether the level of proteasome inhibition achieved with carfilzomib treatment corresponded to clinical response rates, samples from 29 patients with MM who received the highest tolerated dose (20/56 mg/m² in PX-171-007) and the lowest administered dose of carfilzomib (15 or 15/20 mg/m² in PX-171-005) were compared (Fig 3A; Table III). During the first 2 days of carfilzomib treatment (15–20 mg/m²), no statistically significant differences in levels of proteasome inhibition were observed. When the dose was escalated to 56 mg/m² on cycle 1, day 8 for patients in PX-171-007, significantly greater active site occupancy was observed across all three i20S subunits in patients who received the higher dose compared with patients in PX-171-005 who continued to receive the lower dose. In patients receiving 56 mg/m², carfilzomib binding to LMP7, MECL1 and LMP2 was increased by 7·9%, 23·1% and 33·6%, respectively, relative to patients receiving 15 or 20 mg/m² (see Fig 3A and B; $P < 0\cdot05$, $P < 0\cdot001$ and $P < 0\cdot01$, respectively). Increased occupancy of LMP2 at the beginning of C2 was also noted in the high-dose group. Strikingly, 64% of

patients in this analysis who received the higher dose had a clinical response compared with only 11% of patients receiving a lower dose (see Table III). These data suggest a relationship between carfilzomib dose, proteasome inhibition and patient response, in which greater occupancy of multiple immunoproteasome subunits is correlated with an increased likelihood of achieving a clinical response.

Discussion

Proteasome inhibition is a validated strategy for the treatment of B-cell neoplasms, but limitations in conventional assays have prevented pharmacodynamic-based correlative studies of drug action. We describe here the clinical validation and application of ProCISE, a novel assay for monitoring proteasome inhibition via active site occupancy in patient samples that circumvents these limitations and enables a more complete profile of drug action. ProCISE allows for quantitative monitoring of drug binding to all six proteasomal catalytic subunits in cells derived from patient samples, such as PBMC and primary tumour cells. By measuring the binding of each individual subunit, we are able to monitor specific active site inhibition in tumour cells (CD138⁺ myeloma cells) that express the c20S and i20S. This enables monitoring of the pharmacodynamic response to

Fig 3. Immunoproteasome subunit inhibition is correlated with activity in multiple myeloma. (A) Proteasome inhibition in PBMC samples was measured as described in Fig 1. Patients were stratified according to carfilzomib dose: either 20/56 mg/m² from PX-171-007 or 15 and 15/20 mg/m² from PX-171-005. Values (relative to cycle 1, day 1 predose) are presented as mean (±SEM) relative subunit concentration or specific activity. The shaded area represents the time points in which the dose was stepped up to 56 mg/m² for PX-171-007 patients or 20 mg/m² for some PX-171-005 patients. * $P < 0\cdot05$; ** $P < 0\cdot01$; *** $P < 0\cdot001$ by a *t*-test. (B) Data from panel A at cycle 1, day 8 1-h time point. # $P < 0\cdot05$; ## $P < 0\cdot001$; ### $P < 0\cdot01$; by a *t*-test. C, cycle; CT-L, chymotrypsin-like; D, day; LMP, low-molecular mass polypeptide; MECL, multicatalytic endopeptidase complex-like; PBMC, peripheral blood mononuclear cell; SEM, standard error of the mean.

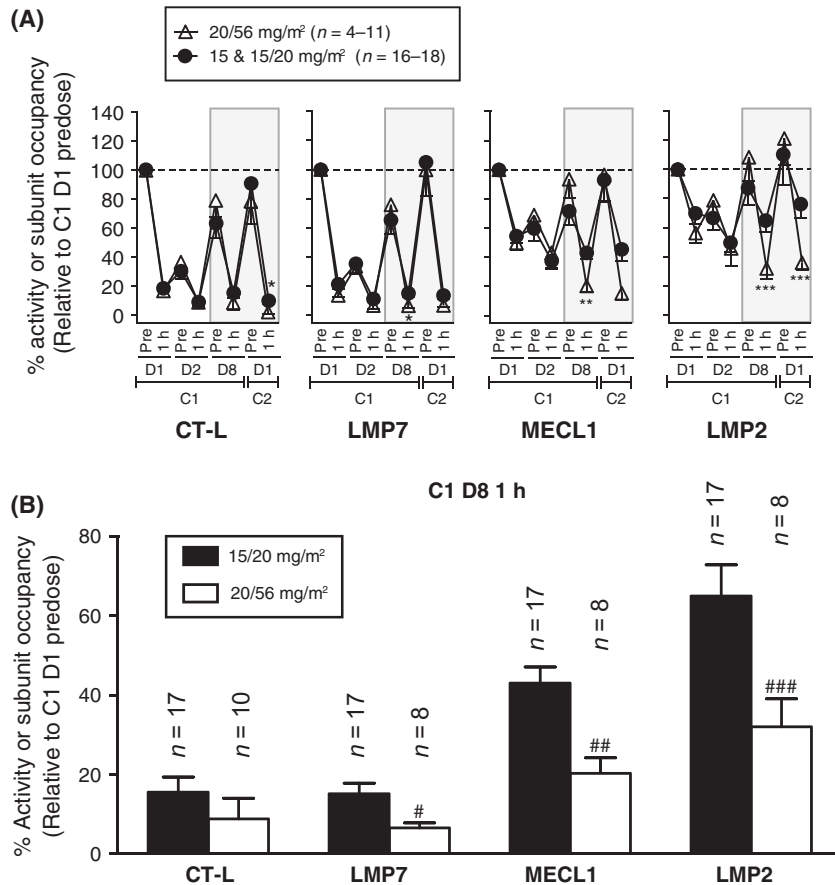


Table III. Clinical response in patients receiving the highest tolerated dose and the lowest administered dose of carfilzomib.*

	20/56 mg/m ² (PX-171-007) (N = 11)	15 or 15/20 mg/m ² (PX-171-005) (N = 18)
Responders (≥ PR)	64% (n = 7)	11% (n = 2)
Non-responders (SD + PD)	36% (n = 4)	89% (n = 16)

PD, progressive disease; PR, partial response; SD, stable disease.

*Patients received carfilzomib at a dose of 15 or 20 mg/m² on days 1 and 2 and a target dose of 56 or 20 mg/m² on days 8, 9, 15 and 16 in trials PX-171-007 and PX-171-005, respectively. Some patients in PX-171-005 did not step up to their target dose (e.g. from 15 to 20 mg/m²) and continued to receive 15 mg/m². Patient responses were determined using International Myeloma Working Group criteria (Badros *et al*, 2013; Papadopoulos *et al*, 2013).

compounds that are selective for individual 20S active sites (Muchamuel *et al*, 2009). As existing substrate-based assays are unable to differentiate between c20S and i20S enzymatic activities for any of the subunits, ProCISE currently represents the only known method to monitor the inhibition of individual subunits.

Previously, the pharmacodynamic profile of carfilzomib was assessed *in vitro* using the ProCISE assay (Parlati *et al*, 2009). In tumour cell lines, carfilzomib has been shown to have equivalent and potent activity against both β5 and LMP7 and to be at least 10-fold less potent against the other subunits of the c20S and i20S (Parlati *et al*, 2009). In the current analysis, we verified the potency of carfilzomib for CT-L active sites *ex vivo* and have extended previous findings in samples derived from carfilzomib-treated patients. In patients treated with doses ≥15 mg/m², on average an inhibition rate of greater than 83% was achieved in β5 and LMP7. As in the *in vitro* studies, there was a correspondence between values derived with ProCISE and those derived from the enzymatic assay for CT-L activity. Slightly greater inhibition was observed in whole blood with the enzymatic assay compared with ProCISE, probably due to a quenching effect of the blood matrix – possibly haemoglobin – on the enzymatic activity and the presence of LMP7 in erythrocytes and PBMCs (whole blood samples were not depleted of PBMCs prior to the assay). However, there was a significant correlation between inhibition of CT-L activity and ProCISE in whole blood and PBMCs for the aggregate of paired samples (Table SII). Inhibition levels were also equivalent in patients with MM receiving 20 mg/m² carfilzomib as a 2- to 10-min or 30-min infusion, which is consistent with results reported in animals (Yang *et al*, 2011). It is noteworthy that the overall level of CT-L inhibition seen with carfilzomib is higher than the 65–70% inhibition rate reported in bortezomib-treated patients (Orlowski *et al*, 2002; Papandreou *et al*, 2004; Moreau *et al*, 2008). Using ProCISE, we also demonstrated that the administration of carfilzomib inhibits proteasome

subunit occupancy in bone marrow-derived tumour cells. It is possible that the small sample size (*n* = 5) accounts for discrepancies seen between inhibition levels of CT-L activity assessed by LLVY-AMC and by β5 and LMP7 occupancy assessed by ProCISE; however, we cannot rule out altered binding properties within tumour cells. Nevertheless, these data represent the first known direct correlation between proteasome inhibition in blood samples to that in tumour cells in patients with MM.

Clinical trials have demonstrated that carfilzomib and bortezomib are clinically active regardless of the degree of renal insufficiency in patients (Jagannath *et al*, 2005; San-Miguel *et al*, 2008; Badros *et al*, 2013). Consistent with this, we found that carfilzomib induces consistent and potent inhibition of multiple proteasomal active sites in MM patients, regardless of patient renal function. In addition, we found that concomitant drug administration did not affect levels of active site occupancy, as assessed in patients receiving carfilzomib in combination with varying doses of lenalidomide. To our knowledge, this is the first time the subunit-specific pharmacodynamic activity of a proteasome inhibitor has been characterized in MM patients with renal impairment or concomitant drug administration, providing additional information on the activity of this class of agents in a variety of clinical settings.

In rodents, proteasome activity following treatment with carfilzomib recovers with a half-life of approximately 24 h in tissues (e.g. adrenal and bone marrow), but prolonged inhibition was observed in whole blood for which recovery is dependent on red blood cell turnover (Demo *et al*, 2007). Little to no recovery of proteasome activity was observed in whole blood following the administration of carfilzomib, which is consistent with our preclinical results. Somewhat surprisingly, recovery of activity in PBMCs was slower than predicted based on animal data. Minimal recovery of proteasome activity was observed 24 h after the first dose, and incomplete recovery was seen after the 5-day non-dosing period during the first week of treatment. This effect is unlikely to be due to prolonged exposure, because carfilzomib is rapidly cleared from the plasma (O'Connor *et al*, 2009). Rather, these data demonstrate that carfilzomib results in proteasome inhibition that lasts at least 48 h and suggests that PBMCs have an altered rate of proteasome recovery relative to other cell types. In contrast, a 50% recovery of activity was seen 12 h after the administration of bortezomib, with full recovery occurring 36 h after dosing (Hellmann *et al*, 2011). We hypothesize that differences in proteasome recovery between the two drugs may be due to the irreversible binding of carfilzomib in contrast to the reversible binding of bortezomib to the proteasome (Demo *et al*, 2007).

Interestingly, administration of carfilzomib resulted in increased occupancy of MECL1 and LMP2 in PBMCs but not of β1 and β2 in whole blood. This finding was unexpected given the weak potency of carfilzomib *ex vivo* against non-CT-L subunits in whole blood and PBMCs. Levels of MECL1 and LMP2 inhibition have a statistically significant

linear dose effect in which higher doses of carfilzomib lead to greater inhibition. While inhibition of LMP2 in samples from bortezomib-treated patients has previously been reported (Kraus *et al*, 2007; Arastu-Kapur *et al*, 2011), this is the first known report of MECL1 inhibition by a proteasome inhibitor in patients. The mechanism of selective LMP2 and MECL1 inhibition by carfilzomib relative to $\beta 1$ and $\beta 2$ inhibition remains to be defined. Although data from our group demonstrated that combined inhibition of $\beta 5$ and LMP7 was necessary and sufficient to induce apoptosis in myeloma cells *in vitro* (Parlati *et al*, 2009), several studies have postulated a role for the other active sites of the i20S in anti-myeloma responses. Kuhn *et al* (2009) have shown that LMP2-selective inhibitors can induce myeloma cell death, and multiple reports have demonstrated synergistic tumour cell killing with carfilzomib or bortezomib and either an LMP2/ $\beta 1$ or MECL1/ $\beta 2$ selective inhibitor (Britton *et al*, 2009; Mirabella *et al*, 2011). It is possible that the simultaneous inhibition of multiple i20S subunits contributes to the potent anti-tumour activity of carfilzomib in patients with MM, including patients that are refractory to bortezomib (Siegel *et al*, 2012; Vij *et al*, 2012a), particularly given that approximately 75% of the proteasomes in isolated myeloma cells are i20S.

Importantly, we observed a relationship between carfilzomib dose, i20S inhibition, and clinical response in patients with MM. Patients receiving a dose of 56 mg/m² of carfilzomib beginning on day 8 showed significantly greater inhibition of all three i20S subunits compared with patients who received 15 or 20 mg/m² of carfilzomib. Given that near-complete inhibition of LMP7 was noted even at 15 mg/m², higher doses of carfilzomib resulted in small differences in LMP7 inhibition. However, significantly greater levels of inhibition of both LMP2 and MECL1 were noted in patients receiving 56 mg/m² of carfilzomib compared with those receiving 15 or 20 mg/m² (i.e. cycle 1, day 8). At cycle 2, day 1, LMP2 inhibition remained significantly different between the two groups, reaffirming that higher doses of carfilzomib led to greater i20S inhibition. The difference observed in MECL1 at cycle 2, day 1 is probably not statistically significant because of the small sample size ($n = 4$) in the 20/56-mg/m² cohort. Of the patients analysed for pharmacodynamics, 64% of patients receiving 20/56 mg/m² achieved a clinical response compared with only 11% of patients receiving 15 or 20 mg/m². While these samples were derived from different patient populations, both trials enrolled patients with relapsed and/or refractory MM who had been treated with at least two prior regimens. Overall, these data suggest that higher doses of carfilzomib lead to greater levels of i20S inhibition, resulting in improved clinical responses in patients with MM. Although these data are derived from small numbers of patients and should be verified in larger, randomized trials, they do support previous findings of a dose-response relationship seen with earlier phase II trials of single-agent carfilzomib (Squifflet *et al*, 2011).

In summary, we present the first clinical validation and application of the ProCISE assay. Administration of

carfilzomib results in potent and prolonged inhibition of multiple proteasome active sites in patients with MM. In patients with MM, there is a dose-dependent relationship between pharmacodynamic inhibition and anti-tumour response following carfilzomib treatment. It is worth noting that the ProCISE assay can also be used to assess and compare the activity of other proteasome inhibitors besides carfilzomib (Parlati *et al*, 2009), as ProCISE is not treatment-specific. Therefore, ProCISE represents a potentially valuable new tool for measuring the activity of proteasome inhibitors in clinical trials and can be used to relate drug action to patient outcomes.

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Author contributions

SJL, KL and FP designed and performed research, collected data, and analysed and interpreted data. MKB, SAK and CJK designed research and analysed and interpreted data. LK performed statistical analysis. TFW and AFW collected data. KPP, RN, AZB, RV, SJ, DS, MW and GJA collected data and provided clinical samples. All authors contributed to the writing of this manuscript and approved it for submission.

Conflict of interest

SJL, KL, FP, MKB, SA-K, LK, TFW, AFW and CJK were employees of Onyx Pharmaceuticals at the time of this work. RV has received research funding from Onyx Pharmaceuticals and Celgene, has participated in speaker bureaus for Celgene, Millennium and Onyx Pharmaceuticals, and has received honoraria from Eli Lilly and Bristol-Myers Squibb. DS has served as a consultant, participated in advisory boards and received honoraria from Millennium and Celgene. MW has received research funding from Onyx Pharmaceuticals, Celgene, Millennium, Pharmacyclics, Janssen-Cilag and Novartis, and received honoraria from Onyx Pharmaceuticals and Celgene. SJ has received honoraria from Onyx Pharmaceuticals, Millennium, Celgene and Merck, and is on the board of or advisory committees for Ortho Biotech, Imdex, Medicom WorldWide, OptumHealth Education and PER Group. KPP has served as a consultant for Proteolix and has received research funding from Onyx Pharmaceuticals and Proteolix. RN has served as a consultant and received research funding from Onyx Pharmaceuticals,

Celgene and Millennium, and has participated in speakers bureaus for Celgene and Millennium. GJA and AZB have no conflicts of interest to disclose.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1: Methods and Results

Table SI. ProCISE precision and interday variability.

Table SII. Correlation of the relative change of enzymatic inhibition of chymotrypsin-like active site occupancy and β 5/LMP7 active site occupancy in whole blood and PBMC samples.

Fig. S1. Validation of the ProCISE assay.

Fig. S2. Potency of carfilzomib in ex vivo whole blood and PBMC.

Fig. S3. Dose-dependent immunoproteasome inhibition.

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