Proteasome Inhibition Measurements: Clinical Application

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Background: PS-341, a selective inhibitor of the proteasome, currently is under evaluation as an anticancer agent in multiple phase I clinical trials. In animal-model studies, PS-341 was rapidly removed from the vascular compartment and distributed widely, quickly approaching the limits of detection. An accurate pharmacodynamic assay has been developed as an alternative or complement to pharmacokinetic measurements.

Methods: Fluorogenic kinetic assays for both the chymotryptic and tryptic activities of the proteasome have been optimized for both whole blood and blood cells. Using the ratio of these activities and the catalytic mechanism of the proteasome, we developed a novel method of calculating percentage of inhibition, using two structurally unrelated inhibitors (PS-341 and lactacystin).

Results: This ratio method was demonstrated to be sensitive (detection limit of 13% inhibition with 10 μg of cell lysate), specific to the proteasome (PS-341 provides >98% inhibition), accurate (112% analyte recovery), and precise (0% ± 5% inhibition at 0 nmol/L PS-341 and 74.5% ± 1.7% inhibition at 200 nmol/L PS-341). Using these assays, we found that both erythrocytes and leukocytes contain proteasome at 3 μmol/L. Pharmacodynamic results for PS-341 obtained from the whole-blood ratio method were comparable to those using leukocytes determined by another method.

Conclusions: The described assay provides a reliable method for studying the pharmacodynamics of proteasome inhibitors and is now in use in concurrent phase I clinical trials with PS-341.

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The ubiquitin-proteasome pathway is essential for the regulation of numerous cellular proteins, including those mediating inflammatory conditions and cancer (1–6). Proteins that are destined for proteolysis are first tagged with polyubiquitin chains through a cascade of enzyme-catalyzed events. These “marked” proteins are then degraded via the 26S proteasome in an ATP-dependent manner (7). The 26S proteasome (EC 3.4.99.46) is a large, multisubunit enzyme (Mr = 2 000 000) found in high concentration in all mammalian cells (Fig. 1). The ATP hydrolytic activity and the subunits that bind ubiquitin in the 26S proteasome are contained within a protein complex known as the 19S cap. The ATP-independent proteolytic activity of the proteasome is contained within the central 20S core (Mr = 730 000). The 20S proteasome is a multicatalytic protease that has three well-characterized peptidase activities—chymotryptic, tryptic, and postglutamyl peptide hydrolytic—that are associated with three distinct subunits: β5*, β2*, and β1*, respectively (8). Each site is defined by its ability to hydrolyze peptide substrates in vitro, with hydrophobic, basic, or acidic amino acids in the P1 position.

Inhibitors of the proteasome have been designed based on either the natural product, lactacystin (9), or synthetic peptidyl derivatives (10). PS-341, a peptidyl boronic acid, is a novel inhibitor of the chymotryptic site within the 20S proteasome (11). This molecule shows at least 500-fold selectivity for the proteasome over other enzymes and receptors and exhibits substantial potency in cell-free and in vitro cell-based assays (MDS PanLabs, Bothell, WA, unpublished results). PS-341 has shown significant activity in preclinical murine tumor models (3) where the antitumor activity was positively correlated with the degree of proteasome inhibition measured both in leukocyte and tumor biopsy material. PS-341 at 1 mg/kg body weight produced an ~60% reduction in the growth of PC-3 tumors in mice, which correlated with ~85% inhibition of proteasome activity in leukocyte lysates.

PS-341 currently is under evaluation as an anticancer agent in multiple phase I clinical trials in which blood concentrations of the drug are being measured by liquid
chromatography–mass spectrometry to determine standard pharmacokinetic parameters (R.A. Newman and P.J. Elliott, personal communication). Based on animal models (including nonhuman primates), the drug is distributed rapidly and falls to near detection limits within minutes of intravenous dosing (P.J. Elliott, unpublished results). Whether this pattern will carry over to the clinical trials at the highest dose tolerated is still unknown. The pharmacodynamic profile of the drug could also be evaluated to assess the activity of PS-341 at its target site, the proteasome. As such, it would be possible not only to evaluate blood concentrations of PS-341 but also to record the extent of enzyme inhibition over time.

To explore the possibility that the proteasome activity assay, or a variation of it, could be used in future clinical studies, the present series of experiments were undertaken. The ex vivo assays reported here were developed utilizing the current knowledge of the catalytic activities within the 20S proteasome and a previous report by Stein et al. (12) on proteasome activity measurements. The focus of the method development was to obtain a simple assay that was both rapid and reproducible and could be used to determine accurately the proteasome activity in rodent blood samples treated with PS-341. To confirm that the tryptic and chymotryptic activities were attributable only to the proteasome, a proteasome inhibitor from a second structurally unrelated class was also used, clasto-lactacystin-β-lactone (lactacystin). Using multiple assays, we cross-validated each format and determined the optimal assay methodology. Herein, we describe the development of these pharmacodynamic assays to record proteasome activity.

Materials and Methods

Materials

The fluorometers used were an F-2000 and an F-4500 fluorescence spectrophotometer (both from Hitachi) containing a jacketed cuvette holder for temperature control. Reaction temperatures were maintained using a VWR Scientific Model 1166 circulating water bath. Rates were taken from collected data using Lab Calc (Galactic Industries) or Mac Lab Chart, Ver. 3.5.4 (AD Instruments). Initial velocities were fitted by nonlinear least-squares analysis to standard equations (13) using Jandel Scientific SigmaPlot, Ver. 4.17 for Macintosh.

Male Sprague–Dawley rats (180–220 g) were obtained from Harlan. All animal procedures were approved by the Institutional Animal Use and Care in accordance with NIH guidelines. Heparinized blood was obtained from anesthetized (8 mg of ketamine plus 1.2 mg of xylazine per 100 g of body weight) rats by cardiac puncture. Heparinized human blood was obtained from Millenium volunteers. 20S proteasome was purified from rabbit reticulocytes by published methods (12).

Stock solutions of PS-341 and clasto-lactacystin β-lactone (lactacystin) were prepared in dimethyl sulfoxide (DMSO) for all in vitro studies. The final concentration of DMSO in all incubations was <1.0%. For in vivo work, PS-341 was prepared in vehicle (980 mL/L saline solution, 20 mL/L ethanol, 5 g/L ascorbic acid).

Preparation of Blood Cell Lysates from Rat and Human Blood

Venous blood (10 mL) was collected from human subjects into heparin-containing tubes and mixed, in accordance with the manufacturer’s instructions. For rat samples, 20 µL of heparin was mixed into 2 mL of collected venous blood. Blood samples were prepared according to the method of Adams et al. (3) with the following modifications: packed whole blood (PWB) lysates were prepared without the intervening Nycoprep separation, and red blood cell (RBC) lysates were prepared by collecting the pellet from the Nycoprep separation, with careful avoidance of the granulocyte layer. Complete lysis was achieved within 15 min.

Assay of Proteasome Activities in Blood Lysates

Kinetic measurement of proteasome activity followed that described by Adams et al. (3). PWB or RBC lysate (20–100 µg of protein) or white blood cell (WBC) lysate (>10 µg) was added to the cuvette. When the proteasome chymotryptic activity in PWB or RBC lysates was measured, the same assay buffer was used except that the sodium dodecyl sulfate (SDS) concentration was 0.5 g/L, except as noted. The signal was linear between 3 and 60 min. Measurement of the proteasome tryp tic activity for all lysate types was performed in 60 µmol/L Bz-Val-Gly-Arg-7-amido-4-methylcoumarin (VGR-AMC) in 20 mmol/L HEPES, pH 8.0, containing 0.5 mmol/L EDTA and 10 mL/L DMSO. Enzyme units are expressed as pmol AMC/s. This is equivalent to 6 × 10⁻³ U.

Determination of the Michaelis-Menten Constants

Concentrations of the substrate Suc-Leu-Leu-Val-Tyr-AMC (LLVY-AMC; chymotryptic activity) were evaluated in 5 or 10 µmol/L increments up to 100 µmol/L. VGR-AMC (tryp tic activity) was evaluated in a similar manner up to 400 µmol/L except that no SDS was included in the buffer.

Inhibition of Proteasome Chymotryptic and Tryptic Activities in Human Blood Lysate by Lactacystin in Vitro

Inhibitor (lactacystin, 0–4 µmol/L; or PS-341, 0–1 µmol/L) was added to 30–50 µL of blood lysate (protein

1 Nonstandard abbreviations: DMSO, dimethyl sulfoxide; PWB, packed whole blood (all circulating blood cells); RBC, red blood cell; WBC, white blood cell (mononuclear cell); SDS, sodium dodecyl sulfate; AMC, 7-amido-4-methylcoumarin; LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-AMC; and VGR-AMC, benzoyl-Val-Gly-Arg-AMC.
concentration, 1.3–7 g/L) and incubated at 37 °C for 30 min. The samples were then placed on ice and assayed within 6 h. Proteasome activities were measured as described above. The tryptic activity data for PS-341 were fitted to the equation for general nonessential activation (13).

**EFFECT OF PS-341 ON THE CHYMOTRYPIC AND TRYPIC ACTIVITIES IN PWB LYSATES PREPARED FROM PS-341 IN VIVO-TREATED RATS**

Rats received intravenously injections of 0–0.3 mg/kg PS-341 in 100 μL of vehicle (980 mL/L saline, 20 mL/L ethanol, 5 g/L ascorbic acid). After 1 h, heparinized blood was obtained, and WBC and PWB lysates were prepared for the chymotryptic and tryptic assays as described above.

**Results**

The chymotryptic activity of the 20S proteasome typically is measured in vitro by monitoring the release of the fluorophore, AMC, from the synthetic peptide substrate, LLVY-AMC in the presence of SDS (12). The following studies were undertaken to improve this assay to accurately measure proteasome activity and the extent of inhibition by specific tight-binding proteasome inhibitors in biological material from both preclinical and clinical laboratories. The assay development used the inhibitors PS-341 and lactacystin, which elicit complete inhibition of the chymotryptic activity within the 20S core (Figs. 1 and 2). By contrast, it was found that PS-341 also induces activation of the tryptic catalytic activity within the 20S proteasome, whereas lactacystin produces complete inhibition of the tryptic activity, as determined using a specific peptide substrate for this hydrolytic site, VGR-AMC (Fig. 2).

When we used the published method to determine the extent of proteasome inhibition, it was necessary to calculate the specific activity of the chymotryptic site in the presence and absence of PS-341 and then apply the following equation (Eq. 10 in the Appendix):

\[
\%I = 100 \times \left(1 - \frac{SpA_I}{SpA_U}\right)
\]

where %I is the percentage of inhibition of the chymotryptic activity of the 20S proteasome, \(SpA_I\) is the chymotryptic specific activity of the proteasome in the presence of inhibitor, and \(SpA_U\) is the chymotryptic specific activity of the proteasome in the absence of inhibitor.

![Fig. 1. Binding of PS-341 to the proteasome.](image)

PS-341 binds to the chymotryptic subunit of the proteasome (β5*), leading to full inhibition of the chymotryptic activity toward peptides. In contrast, PS-341 activates the tryptic activity toward peptides. Inhibition of the chymotryptic activity of the proteasome leads to full inhibition of ubiquitinated protein hydrolysis.

![Fig. 2. Effect of PS-341 on both chymotryptic and tryptic activity of purified 20S proteasome (A) and inhibition of both chymotryptic and tryptic activities from human WBC lysate by lactacystin (B).](image)

(A), 20S proteasome (47 nmol/L) was incubated with 0.2 g/L of the proteasome activator PA-28 and various amounts of the inhibitor PS-341. The chymotryptic data (□) were fitted to \(v = v_o/(1 + K_{a,app})\), where \(v_o = 49 \pm 3\) fmol AMC/s, and \(K_{a,app} = 24 \pm 5\) nmol/L PS-341. The tryptic data ( ■) were fitted to \(v = v_o/(1 + K_{a,app})\), where \(v_o = 32.0 \pm 0.9\) fmol AMC/s; \(K_{a,app} = 22 \pm 11\) nmol/L PS-341; and \(\alpha = 1.39 \pm 0.05\). Relative velocity was calculated by dividing the observed velocity by the velocity in the absence of PS-341. Both assays were done at 10 μmol/L substrate using 0.35 g/L SDS. Inset, structure of PS-341. (B), the chymotryptic activity ( □) was assayed at 60 μmol/L LLVY-AMC and 0.35 g/L SDS. The tryptic activity ( ■) was assayed at 60 μmol/L VGR-AMC in the absence of SDS. The data were fitted to \(v = v_o/(1 + K_{a,app})\). The parameters for the chymotryptic activity are \(v_o = 380 \pm 20\) fmol AMC/s, and \(K_{a,app} = 0.17 \pm 0.03\) μmol/L lactacystin. The parameters for the tryptic activity are \(v_o = 104 \pm 4\) fmol AMC/s, and \(K_{a,app} = 0.53 \pm 0.08\) μmol/L lactacystin. The relative rate is the measured velocity divided by the calculated \(v_o\). Inset, structure of lactacystin.
This required determination of the total protein concentration of the biological samples being assayed for proteasome activity. Such methodology was reliant on a constant number of proteasomes/mg of protein in a sample. However, the proteasome/protein ratio in a sample may vary depending on tissue type or reproducibility of ex vivo sample preparation. As such, determination of the extent of proteasome inhibition using specific activity measurements might be compromised.

To accurately determine the extent of proteasome inhibition, it was desirable to determine the total proteasome activity in a sample, regardless of whether PS-341 is bound, so that the percentage of inhibition could be determined without the requirement for a separate uninhibited “control” sample. This was achieved by exploring the activity of the other catalytic sites within the proteasome. Studies showed that PS-341 did not inhibit the tryptic activity within the proteasome; rather, it enhanced some. Studies showed that PS-341 did not inhibit the activity of the other catalytic sites within the proteasome.

By the selective proteasome inhibitor, PS-341 (5 μmol/L). Under these conditions, the tryptic activity was activated 1.2-fold over the activity measured in the absence of PS-341. In addition, the activity was inhibited 97% with another specific proteasome inhibitor, lactacystin (1 μmol/L). Comparable data were produced using rat PWB lysates (data not shown). Because two structurally distinct proteasome-specific inhibitors gave essentially complete inhibition of the activity, these results strongly support that the measured hydrolytic activity was attributable to the proteasome.

MEASUREMENT OF 20S PROTEASOME ACTIVITY IN PWB LYSATES

Assay methods for the chymotryptic and tryptic activities of the proteasome were developed and optimized for measurement of these activities in PWB lysates. The chymotryptic assay presented here involves the hydrolysis of LLVY-AMC to Suc-Leu-Leu-Val-Tyr-OH and AMC by the chymotryptic activity (associated with subunit β5) of the 20S proteasome. AMC is a highly fluorescent molecule (λex = 380 nm; λem = 440 nm), and its rate of release was measured over time. The addition of 0.5 g/L SDS to the assay buffer (20 mmol/L HEPES, pH 8.0, 0.5 mmol/L EDTA, 60 μmol/L LLVY-AMC, 10 mL/L DMSO) was required to activate the 20S core in PWB lysates (Fig. 3). The exact mechanism by which this occurs is not fully understood, but a recent review details the current hypotheses directed at addressing this issue (7).

When human PWB lysate was used, the chymotryptic activity assay was linearly dependent on total protein between 10 and 100 μg added to a 2-mL assay volume (Fig. 4). The chymotryptic activity was inhibited by 98% by the selective proteasome inhibitor, PS-341 (5 μmol/L).
Two critical modifications were introduced to the 20S proteasome assay reported by Stein et al. (12) to allow for more precise activity measurements in PWB lysates. (a) The SDS concentration in the assay buffer was increased to 0.5 g/L from 0.35 g/L. When 0.35 g/L SDS was used in the assay buffer, the chymotryptic activity was not linear with increasing RBC lysate added to the assay. In fact, addition of RBC lysate under these conditions inhibited the assay (Fig. 3). The source and identity of this endogenous inhibition is not known; however, the presence of such a proteinaceous proteasome inhibitor in RBCs has been reported (14–18). By increasing the concentration of SDS to 0.5 g/L, the inhibitory effects were eliminated, perhaps because of denaturation of the endogenous inhibitor. (b) The concentration of DMSO was maintained at 10 mL/L to improve substrate solubility. At 10 mL/L DMSO, Michaelis-Menten kinetics were observed, which were not attainable at lower DMSO concentrations (12). We did not observe evidence for hysteresis in the assay at 10 mL/L DMSO. Under these new optimal conditions, $V_{\text{max}}$ and $K_m$ were 0.93 ± 0.05 pmol AMC·s⁻¹·mg protein⁻¹ and 13.0 ± 1.9 µmol/L, respectively.

The tryp tic assay presented here involves the hydrolysis of VGR-AMC to Bz-Val-Gly-Arg-OH and AMC by the proteasome (associated with subunit β2*). The by contrast, no SDS was required in this assay buffer (20 mmol/L HEPES, pH 8.0, 0.5 mmol/L EDTA, 60 µmol/L VGR-AMC, 10 mL/L DMSO) because no inhibition of the tryp tic activity was seen by PWB lysate. In addition, it was noted that SDS precipitates the substrate at >10 µmol/L VGR-AMC. When the rat and human PWB lysates were used, the tryp tic assay was linear between 10 and 100 µg of protein added to a 2-mL assay (Fig. 4).

PS-341 cannot be used to demonstrate that the proteasome accounted for all of the tryp tic activity within the PWB lysate because it did not inhibit this activity (Fig. 2A). Lactacystin is a selective inhibitor of the proteasome (9) and provides 94% inhibition of tryp tic activity in human PWB lysate (8 µmol/L). Similar results were obtained for rat PWB lysate. Therefore, all of the tryp tic activity present in rat and human PWB lysate appears to be attributable to the proteasome. The tryp tic activity also follows Michaelis-Menten kinetics with a $V_{\text{max}} = 1.47 ± 0.15$ pmol AMC·s⁻¹·mg protein⁻¹ and $K_m$ (VGR-AMC) = 130 ± 30 µmol/L.

The accuracy of both the chymotryptic and tryp tic assays for human samples was evaluated using an analytical recovery method, i.e., how much of a known amount of enzyme activity was recovered after addition to the appropriate biological sample. This was done by titrating purified 20S proteasome into an assay containing human PWB lysate and then using the slope to calculate the specific activity of the added 20S proteasome (Table 1), effectively subtracting the PWB lysate proteasome activity. The recoveries of the chymotryptic and tryp tic activities when assayed in the presence of PWB lysate were 131% and 117%, respectively. More importantly, the ratio of the two activities, $v_C/v_T$, used in the calculation of percentage of inhibition in Eq. 8, in the presence of PWB lysate gave a more accurate recovery of 112% (3.4 ± 0.5 for purified 20S; 3.8 ± 0.2 in PWB lysate), demonstrating that the chymotryptic-to-tryp tic ratio method is more accurate.

As an indication of the analytical sensitivity, the specific activity method has a calibration curve slope (%I vs. $S$) of −80 and the ratio method has an approximate calibration curve slope (%I vs. $v_C/v_T$) of −24. Thus, for a human PWB lysate, 0%, 30%, and 80% inhibition would correspond to specific activities of 1.25, 0.88, and 0.25 pmol AMC·s⁻¹·mg protein⁻¹, respectively. For the ratio method, a human PWB lysate at 0%, 30%, and 80% inhibition would give $v_C/v_T$ values of 3.96, 2.59, and 0.66, respectively.

**Table 1.** Titration of purified 20S proteasome in chymotryptic and tryp tic assays with or without human PWB lysate.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Chymotryptic</th>
<th>Tryptic</th>
</tr>
</thead>
<tbody>
<tr>
<td>20S alone</td>
<td>1.01 ± 0.03</td>
<td>0.30 ± 0.4</td>
</tr>
<tr>
<td>+PWB lysate</td>
<td>1.32 ± 0.03</td>
<td>0.352 ± 0.012</td>
</tr>
<tr>
<td>+WBC lysate</td>
<td>2.347 ± 0.017</td>
<td>1.093 ± 0.009</td>
</tr>
</tbody>
</table>

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**Table 1.** Titration of purified 20S proteasome in chymotryptic and tryp tic assays with or without human PWB lysate.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Specific activity, nmol AMC·s⁻¹·mg protein⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>20S alone</td>
<td>1.01 ± 0.03</td>
</tr>
<tr>
<td>+PWB lysate</td>
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**Table 1.** Titration of purified 20S proteasome in chymotryptic and tryp tic assays with or without human PWB lysate.

- Assayed at 60 µmol/L LLVY-AMC, 0.35 g/L SDS otherwise indicated.
- Assayed at 60 µmol/L VGR-AMC, no SDS.
- Assayed at 60 µmol/L LLVY-AMC, 0.5 g/L SDS.

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**Table 1.** Titration of purified 20S proteasome in chymotryptic and tryp tic assays with or without human PWB lysate.
tryptic activity (complete inhibition at 7 μmol/L lactacystin) in human WBC lysates, with similar results in rats (data not shown). The chymotryptic activity from human WBC lysate followed Michaelis-Menten kinetics with a \( V_{\text{max}} = 35 \pm 1 \text{ pmol AMC} \cdot \text{s}^{-1} \cdot \text{mg protein}^{-1} \) and \( K_m \) (LLVY-AMC) = 30.3 ± 1.8 μmol/L. A Michaelis constant for VGR-AMC in the tryptic activity from human WBC lysate could not be determined because of substrate insolubility even at 10 mL/L DMSO.

The analytical recovery of chymotryptic activity from purified 20S proteasome added to WBC lysate was 230%; the recovery of tryptic activity was 360%. The ratio of the two assays, \( v_c/v_T \), in the presence of WBC lysate was 63% of that determined in the absence of WBC lysate (3.4 ± 0.5 for purified 20S, 2.15 ± 0.03 in WBC lysate). The activation effect that WBC lysate has on the 20S proteasome is not critical because the percentage of inhibition will be calculated using \( k_C/k_T \) determined for WBC lysate. Cross-validation of results from WBC lysate with those from PWB lysate demonstrated that the methods gave accurate percentages of inhibitions (see below). The specific activity method had a calibration curve slope (\%I vs SpA) of −3.9, and the ratio method had an approximate calibration curve slope (\%I vs \( v_c/v_T \)) of −30.

DETECTION LIMITS AND PRECISION

To determine the detection limits for these assays, the data from the comparison of the four assay formats with blood samples obtained from PS-341-treated rats were used (Fig. 5 and Table 2). We evaluated the minimum percentage of inhibition that could be determined using a 10-μg protein sample for our determination of the detection limits. This provides the most critical evaluation of limitations of the assay because the ability to distinguish between two relatively similar rates at lower protein concentrations is the most difficult to achieve. The percentage of inhibition is more appropriate than drug concentration because this assay is a pharmacodynamic assay. The detection limits for rat PWB lysate methods, determined as 3 SD at a dose of 0.03 mg/kg (Fig. 5), was 17% inhibition for the specific activity method or 13% inhibition for the ratio method. The detection limit for WBC lysate was similarly 16% inhibition for the specific activity method or 13% inhibition for the ratio method.

The precision of inhibition results from the human PWB lysate ratio method is presented in Table 3. The CVs of both the chymotryptic and tryptic assays at 0, 10, 50, and 200 nmol/L PS-341 were all between 2.8% and 6.2% over the 20 measurements (data not shown). The concentrations of PS-341 correspond to the following percentages of inhibitions ± SD: 0 nmol/L, 0% ± 5%; 10 nmol/L, 14% ± 4%; 50 nmol/L, 43% ± 3%; and 200 nmol/L, 74.5% ± 1.7%. Therefore, in vitro assays under these conditions, 10 nmol/L PS-341 represents the detection limit of this assay.


**Table 2. Parameters from chymotryptic:tryptic ratio method.**

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>( k_C/k_T )</th>
<th>( \alpha_T )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure 20S</td>
<td>Rabbit</td>
<td>3.28 ± 0.11</td>
<td>1.66 ± 0.14</td>
</tr>
<tr>
<td>WBC lysate</td>
<td>Human</td>
<td>3.23 ± 0.04</td>
<td>1.33 ± 0.05</td>
</tr>
<tr>
<td>PWB lysate</td>
<td>Human</td>
<td>3.96 ± 0.05</td>
<td>1.24 ± 0.05</td>
</tr>
<tr>
<td>WBC lysate</td>
<td>Rat</td>
<td>2.57 ± 0.05</td>
<td>1.21 ± 0.09</td>
</tr>
<tr>
<td>PWB lysate</td>
<td>Rat</td>
<td>2.29 ± 0.08</td>
<td>1.45 ± 0.12</td>
</tr>
</tbody>
</table>

PS-341 (0.1–1 μmol/L) was titrated into purified 20S proteasome, WBC lysate, or PWB lysate, and incubated for 30 min at 37 °C. Chymotryptic activities were determined using 60 μmol/L LLVY-AMC at 0.35 g/L SDS (pure 20S and WBC) or 0.05 g/L SDS (PWB). Tryptic activities were determined using 60 μmol/L VGR-AMC, no SDS. Percentage of inhibition was calculated by Eq. 10, where SpA is the specific activity of the chymotryptic assay. Percentage of inhibition (\%I) was then fitted to the ratio of the chymotryptic and tryptic activities (\( k_C/k_T \)) by Eq. 8 to calculate the parameters \( k_C/k_T \) and \( \alpha_T \). These parameter values were used throughout this report as indicated in Materials and Methods.
cross-validate each method. In addition, these data demonstrate that the presence of the endogenous inhibition in RBCs does not impact the pharmacodynamic behavior of PS-341 in RBCs when assayed under the described conditions. As such, variations in the endogenous inhibition should not impact the interpretation of PS-341 assay results.

ASSAY ROBUSTNESS

In a clinical setting, the degree of proteasome inhibition in blood could be evaluated in analytical laboratories distant from the collection site. In such cases, it is important to understand the limitations placed on the sample during transit, whether shipped on ice or frozen. Studies may require the use of WBC or PWB samples. Additionally, these lysed samples may need to be frozen and subsequently reevaluated. The robustness of the WBC and PWB assays, under these various conditions, was evaluated.

The stability of the proteasome inhibition by PS-341 in PWB lysates over time was assessed. Human PWB titrated with PS-341 was stored at 0 °C, and PWB lysates were prepared and subsequently assayed at 1, 20, and 42 h. No differences were observed in the behavior of the assays over these time points (Table 4). Therefore, PS-341-treated whole blood can be stored on ice for at least 42 h after collection before analysis begins. Similarly, shipment of samples on dry ice did not adversely affect the analysis (R.A. Newman and P.J. Elliott, personal communication).

Contamination during sample preparation could also be a key source of variability. The following sample preparation reagents were evaluated for their ability to affect the 20S activity in WBC lysates (concentration in assay buffer): Nycoprep Cell Separation Media (5 mL/L), phosphate-buffered saline (5 mL/L), heparin (25 USP units/mL), DMSO (5 mL/L), and EDTA (2.5 μmol/L). No significant effect was seen in either the tryptic or the chymotryptic activity.

Another source of variability for WBC lysate assays could be contamination by RBC lysate components. Only high concentrations of tested components produced any effects in our assays. Data showed that rat RBC lysate (17.4 μg) provided 33% inhibition of rat WBC lysate activity (using 3.2 μg of protein). Hemoglobin and methemoglobin (>12 μg) were found to activate the chymotryptic activity (40%). Bilirubin and biliverdin (0.3 μmol/L) were without effect. Hemin (0.3 μmol/L) provided 49% inhibition. However, complete conversion of 40 μg of hemoglobin would be required to produce this much hemin, an improbability given that typically only 20 μg of total protein was assayed. Although changes in the assays were seen, these conditions are unlikely to be encountered in biological samples and should be of little concern to future studies.

We examined repetitive freeze-thaw cycles of the PWB samples because reevaluation of samples may be necessary over time. The samples were measured for chymotryptic and tryptic activity over eight freeze-thaw cycles. The addition of 100 mL/L glycerol to the sample before freezing dramatically stabilized the activities over the freeze-thaw cycles: chymotryptic activity in the absence of glycerol was highly variable (CV = 24%), but much less variable with 100 mL/L glycerol (CV = 7%), and tryptic activity was even more dramatically affected (CV = 43% vs 6%). Because 100 mL/L glycerol could interfere with the hypotonic lysis of blood cells, it was added after cell lysis.

WBC and PWB samples from a homogeneous rat population provided equivalent and constant data in all four assay variants (Fig. 5). To examine the effect of a heterogeneous human population on assay parameters, we tested for the variance in healthy subjects taken at random, by examining the specific activity of the proteasome and the ratio of the chymotryptic to tryptic activity between and within eight human volunteers (five males, three females; age range, 25–45 years) on six occasions over 6 months. Results indicated that there was little

### Table 3. Precision of chymotryptic-to-tryptic ratio method with PWB cell lysate.

<table>
<thead>
<tr>
<th>PS-341, nmol/L</th>
<th>Mean (v_C/v_T)</th>
<th>Total SD</th>
<th>Total CV, %</th>
<th>Within-run SD</th>
<th>Within-run CV, %</th>
<th>Between-day SD</th>
<th>Between-day CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.27</td>
<td>0.16</td>
<td>6.9</td>
<td>0.08</td>
<td>3.7</td>
<td>0.14</td>
<td>6.0</td>
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<tr>
<td>10</td>
<td>1.88</td>
<td>0.11</td>
<td>6.0</td>
<td>0.12</td>
<td>6.2</td>
<td>0.06</td>
<td>3.1</td>
</tr>
<tr>
<td>50</td>
<td>1.15</td>
<td>0.06</td>
<td>5.0</td>
<td>0.03</td>
<td>2.5</td>
<td>0.05</td>
<td>4.7</td>
</tr>
<tr>
<td>200</td>
<td>0.48</td>
<td>0.03</td>
<td>7.2</td>
<td>0.02</td>
<td>4.7</td>
<td>0.03</td>
<td>6.3</td>
</tr>
</tbody>
</table>

* A single sample of human PWB lysate was split into four samples, and each sample was treated with PS-341 at the indicated concentration (final, 10 mL/L DMSO, 100 mL/L glycerol). Each sample was then incubated at 37 °C for 30 min, aliquoted, and frozen at −80 °C. One sample at each concentration was thawed each day and assayed twice in both the chymotryptic and the tryptic assays over 5 days.

### Table 4. Stability of proteasome inhibition in human PWB lysates during storage on ice.

<table>
<thead>
<tr>
<th>PS-341, μmol/L</th>
<th>Inhibition (%) by (v_C/v_T)</th>
<th>Inhibition (%) by SpA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>0.03</td>
<td>1 12 6</td>
<td>−9 −7 1</td>
</tr>
<tr>
<td>0.10</td>
<td>19 30 25</td>
<td>31 26 27</td>
</tr>
<tr>
<td>0.30</td>
<td>73 73 73</td>
<td>68 72 72</td>
</tr>
<tr>
<td>1.00</td>
<td>94 95 95</td>
<td>93 93 95</td>
</tr>
</tbody>
</table>

PS-341 (in 980 mL/L saline, 20 mL/L ethanol, 1 g/L ascorbic acid) was added to fresh human blood. Final concentration of vehicle in blood, 0.1%. This was incubated for 1 h at 37 °C and then placed on ice. PWB lysate was prepared at 0, 20, or 42 h, and chymotryptic and tryptic activity and protein concentration measurements were made.
intravariation for each subject during the testing period in all four assays. Moreover, the parameters were constant between individuals with SDs between 5% and 14% of the mean (data not shown). For PWB lysate, the mean specific activity for eight volunteers was $1.25 \pm 0.18$ pmol AMC $\cdot s^{-1} \cdot mg$ protein$^{-1}$, and $k_c/k_i$ was $3.49 \pm 0.16$. For WBC lysate, the mean specific activity was $26 \pm 2$ pmol AMC $\cdot s^{-1} \cdot mg$ protein$^{-1}$, and $k_c/k_i$ was $2.10 \pm 0.16$. The values for $k_c/k_i$ for PWB and WBC were different because the chymotryptic activity was assayed under different assay conditions for the different lysate sources (0.5 and 0.35 g/L SDS, respectively). Therefore, day-to-day and individual-to-individual variability of baseline values can be compared to calibrators as a judgement of the validity of results. However, we have found that the determination of $k_c/k_i$ for each individual before dosing is important for accurate assay results. An individual’s $k_c/k_i$ does not vary much with time.

### Discussion

A unique method of measuring proteasome activity has been developed that is sensitive, accurate, and reproducible. This assay not only determines basal proteasome activity in naïve biological material, but can also be utilized to evaluate the effects of drugs that modify such activity. Assay variations were developed for use in whole blood samples or subpopulations of blood cells. As such, this method may allow the determination of the activities of inhibitors at their biological target, the proteasome, and provide a method for studying their pharmacodynamics as an alternative or complement to pharmacokinetic measurements.

The pharmacokinetics of PS-341 currently are under investigation. Preliminary data suggest that it is rapidly removed from the vascular compartment and distributed widely, confirming data using radiolabeled PS-341 (3). As such, it is not yet possible to correlate plasma concentrations of the drug with the degree of proteasome inhibition in blood samples. However, the concentration of the proteasome in blood cells is $\sim 3 \mu mol/L$ (see below), which is high relative to the $K_i$ of PS-341 [0.62 nmol/L, compound 15 (11)]. Hence, it is expected that most of the drug that enters the cell will be bound by the proteasome. The calculations reported here demonstrate that the concentration of proteasome within red or white blood cells is equivalent (see below), and therefore, results from either biological sample should provide comparable data sets. Because the majority of whole blood (PWB) is RBCs (>99%), variations in WBC counts attributable to disease or diurnal rhythms will have insignificant impact on the final result. It should be noted that when using the degree of proteasome inhibition in WBCs as a pharmacodynamic measure over time, one should take into account the biological life span of those cells. If the cell type has a half-life of a few days, it is important to consider this when determining the rate of recovery of proteasome activity. In contrast, the half-life of RBCs is 15–17 weeks.

As such, under circumstances other than those where proteasome inhibition is being investigated in a particular cell type (e.g., in studies of leukemic cells), it is most likely that the simplest sample preparation would be used in the clinic. Thus, samples are likely to be collected, frozen without any preparation, and assayed at a later date. The current data support assays not only from isolated WBC populations but also from whole blood preparations containing mainly RBCs. Although Kahn et al. (19) previously have reported proteasome-like activity under similar assay conditions, this is the first report to clearly demonstrate quantitative proteasome activity in RBC lysates. This is highlighted by the fact that two chemically distinct proteasome inhibitors, PS-341 and lactacystin, both inhibit the chymotryptic activity in RBC lysates, which strongly suggests that these effects are attributable solely to the proteasome.

The methodology developed here is based on the fact that proteasome inhibitors affect the multiple catalytic sites differentially within the 20S core (20). Selection of appropriate peptidic substrates to determine activity at each active site was critical to this study along with the selective inhibitors of such sites. Whereas lactacystin inhibited all three sites (albeit with different affinities), PS-341 was shown to inhibit the chymotryptic activity (β5*) and actually increase tryptic activity. Based on the mechanism by which boronates inhibit serine proteases (21–23) and the mechanism by which aldehydes inhibit the proteasome (24), it is proposed that the boronate group within PS-341 interacts with the active site threonine within the proteasome. Presumably, it is this boronate:threonine binding that confers some conformational change to the trypic subunit β2* to induce the activation. Currently, it is not known whether the other subunits in the 20S core undergo any alteration in response to PS-341 binding to β5*.

The four assay formats, specific activity and the chymotryptic:tryptic ratio method with either PWB or WBC lysates, provided an opportunity to select the simplest, most sensitive, and precise assay to measure proteasome inhibition. In addition, the development of the four assay formats allowed cross-validation of the results. The data presented in Fig. 5 illustrate that the percentage of inhibition, as determined by each assay format, is internally consistent and that each assay will give the same pharmacodynamic result, demonstrating four reliable pharmacodynamic assays.

The variability of proteasome specific activity and the ratio of chymotryptic to tryptic activity between rats from the same population, or even between human volunteers, are quite low (CV, 5–14%). Assays developed with PWB lysates were no more variable than with WBC lysates, and preparation of PWB lysate is simpler and more reproducible than the preparation of WBC lysate. As such, the assays in PWB are likely to be chosen in future studies.

In the validation of this assay, we realized that nearly all of the data necessary to calculate the concentration of
20S proteasome in blood cells had been collected. Because cellular content of the 20S proteasome is high (25), we were interested in how this value would influence our expectations of the pharmacodynamics of PS-341. The concentration of proteasome within a cell can be converted to the 20S proteasome form can be calculated if the specific activity of purified 20S proteasome is known and if there are no interfering inhibitors or activators in the crude lysate. Table 1 demonstrates that this assumption was accurate to within a factor of 2 for the chymotryptic activity. The protein content of blood cells and the specific activity of each sample allowed the cellular content of the 20S proteasome to be determined (Table 5, row 3). The content determined for RBC lysate was lower than the previously determined 0.28% ± 0.04%, which was calculated based on immunoreactivity (25). The source of this discrepancy is not understood, but the consistency of the results detailed below lend confidence to the value derived in this report. The numbers given within this report are within the same range as those given by Tanaka et al. [0.07–1.0% (25)].

Although the proteasome content of several cell types, tissues, and organs has been determined previously (25, 26), the actual cellular concentration of 20S proteasome has been determined only for yeast (27), which has a proteasome concentration in the total cell of 0.4–0.7 μmol/L. Using the data from Table 5 and the indicated values from the literature, we calculated that the cellular concentration of 20S proteasome is 1–6 μmol/L in WBCs and 1.9–4.1 μmol/L in RBCs. Therefore, the concentration of 20S proteasome in WBCs and RBCs is equivalent, and as such, data collected from either cell type should reflect similar proteasome inhibition. Finally, it is unlikely that full inhibition of the proteasome would be observed with PS-341 because of the high cellular proteasome concentrations. Thus, the cellular response to PS-341 will be dictated by the availability of the drug rather than the proteasome concentration within the cell.

In conclusion, a novel approach to determining the degree of inhibition has been applied to the proteasome in blood. The assay consists of measuring proteasome activity at two sites (chymotryptic and tryptic) within the 20S core of the proteasome and determining the degree of inhibition conferred by PS-341. Variations of the assay allow similar data sets to be calculated in subpopulations of blood cells, assuming that certain precautions are taken to avoid RBC contamination. Currently, the assays are being explored in phase I clinical trials to validate the methods and to determine the optimum conditions for collection, storage, and preparation of samples. To date, the assays have provided a source of real-time pharmacodynamics on individual patients dosed with PS-341. In the future, these results may be invaluable for correlation with drug blood concentrations, safety, and clinical activity data. In addition, the resolution of issues around measuring blood cell proteasome inhibition may be applicable to other biological material (e.g., tissue biopsy). Finally, the current cuvette-based assay appears readily adaptable to a high-throughput 96-well plate format.

### Table 5. Calculation of concentration of 20S proteasome in human blood cells.

<table>
<thead>
<tr>
<th></th>
<th>WBCs</th>
<th>RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude specific activitya</td>
<td>21 ± 6</td>
<td>0.93 ± 0.09</td>
</tr>
<tr>
<td>Pure specific activityb</td>
<td>2347 ± 17</td>
<td>1320 ± 30</td>
</tr>
<tr>
<td>Cell 20S contentc</td>
<td>0.9% ± 0.2% (pn/pn)</td>
<td>0.07% ± 0.008% (pn/pn)</td>
</tr>
<tr>
<td>Total protein/cell</td>
<td>68 ± 14 pg</td>
<td>280 ± 50 pg</td>
</tr>
<tr>
<td>20S content/celle</td>
<td>0.6 ± 0.3 pg 20S/cell</td>
<td>0.6 ± 0.3 pg 20S/cell</td>
</tr>
<tr>
<td>Moles 20S/celld</td>
<td>8 ± 4 x 10^-19</td>
<td>2.7 ± 0.8 x 10^-19</td>
</tr>
<tr>
<td>Volume of blood cellf</td>
<td>200–350 fL</td>
<td>85–100 fL</td>
</tr>
<tr>
<td>[20S] in blood cellg</td>
<td>1–6 μmol/L</td>
<td>1.9–4.1 μmol/L</td>
</tr>
</tbody>
</table>

---

a Specific activities were determined from the chymotryptic activity at 60 μmol/L LLVY-AMC in 0.35 g/L SDS (WBCs) or 0.5 g/L SDS (RBCs). Data were taken from blood samples from six human individuals for WBCs and eight human individuals for RBCs. Enzyme specific activity is expressed as pmol AMC · s⁻¹ · mg protein⁻¹.

b From Table 1.

c Cell 20S content times protein/cell.

d 20S content per cell divided by molecular weight of 20S proteasome (731,228 from SWISSPROT).

e Moles 20S per cell divided by volume of blood cell.

f Refs. (28–35).

g Moles 20S/cell divided by molecular weight of 20S proteasome (731,228 from SWISSPROT).


34. Baskurt OK. Deformability of red blood cells from different species studied by resistive pulse shape analysis technique. Biochemistry 1996; 33: 169–79.


Appendix

DERIVATION OF THE EQUATION RELATING CHYMOTRYPTIC TRYPTIC ACTIVITY RATIO TO PERCENTAGE OF INHIBITION BY A PROTEASOME INHIBITOR

It was observed that under conditions where the chymotryptic activity of the proteasome was inhibited by PS-341, the tryp tic activity was not inhibited but activated (see below). Because PS-341 does not inhibit the tryp tic activity, measurement of the tryp tic activity should correlate with the absolute concentration of 20S proteasome in the sample, provided that the activation of the tryp tic activity is taken into account. Thus, measurement of the two activities in a crude sample such as WBC or PWB lysate allows direct calculation of the fraction of proteasome chymotryptic activity sites that are bound by PS-341.

The equations describing how the measurement of the two activities in a lysate treated with PS-341 can be used to determine the fraction of 20S bound by inhibitor are derived below. These equations apply to any enzyme that is taken into account. Thus, measurement of the two activities in a crude sample such as WBC or PWB lysate allows direct calculation of the fraction of proteasome chymotryptic activity sites that are bound by PS-341.

Let $k_C$ and $k_T$ be the apparent rate constants for the chymotryptic and tryp tic sites, respectively, under a defined set of assay conditions (in the absence of inhibitor):

$$v_C = k_C[20S]_0$$

(1)
\[ v_T = k_T[20S]_{TOT} \]  

(2)

where \([20S]_{TOT}\) is the total proteasome concentration.

In the presence of a proteasome modifier (e.g., an inhibitor) that leads to formation of an E·I complex, the rate constant for chymotryptic and tryptic sites may be altered by the single molecule of modifier binding to an unidentified site. This effect can be represented by \(a_C k_C\) and \(a_T k_T\), where \(a = 0\) indicates total inhibition by the modifier (i.e., E·I complex has no activity); \(a < 1\) indicates partial inhibition (i.e., E·I complex is less active than E); \(a = 1\) indicates no inhibition (i.e., E·I complex is as active as E); and \(a > 1\) indicates activation (i.e., E·I complex is more active than E).

At a given fraction of modified proteasome \((f = [E·I]/[E]_{TOT})\):

\[ v_C = k_c [20S]_{TOT} (1 - f) + \alpha_C k_c [20S]_{TOT} (f) \]  

(3)

\[ v_T = k_T [20S]_{TOT} (1 - f) + \alpha_T k_T [20S]_{TOT} (f) \]  

(4)

Then

\[ \frac{v_C}{v_T} = \frac{k_c}{k_T} \left( \frac{1 - f}{1 - f + \alpha_C f} \right) \]  

(5)

and

\[ f = \frac{\left( \frac{k_c}{k_T} - \frac{v_C}{v_T} \right) k_T}{\frac{k_c}{k_T} - \frac{v_C}{v_T} + \alpha_T \frac{v_T}{v_C} - \alpha_C \frac{k_T}{k_c}} \]  

(6)

The parameter \(k_c/k_T\) is experimentally determinable. The parameter \(k_c/k_T\) is dependent on the assay conditions used for the measurement of the chymotryptic and tryptic activities but is not dependent on the nature of the inhibitor. The parameters \(\alpha_C\) and \(\alpha_T\) are dependent on the identity of the inhibitor. Once \(k_c/k_T\), \(\alpha_C\), and \(\alpha_T\) are known for a defined set of assay conditions for a particular inhibitor, the chymotryptic and tryptic activities of a crude sample can be used to calculate the degree of proteasome inhibition by the inhibitor.

In the specific case described in the Results, where \(\alpha_C = 0\):

\[ \frac{v_C}{v_T} = \frac{k_c}{k_T} \left( \frac{1 - f}{1 - f + \alpha_T f} \right) \]  

(7)

\[ \%I = \frac{100 \times \left( \frac{k_c}{k_T} - \frac{v_C}{v_T} \right)}{\left( \frac{k_c}{k_T} - \frac{v_C}{v_T} + \alpha_T \frac{v_T}{v_C} \right)} \]  

(8)

or

\[ 100 \times \left( \frac{k_c}{k_T} - \frac{v_T}{v_C} - 1 \right) \]  

(9)

where \(\%I\) is the percentage of inhibition of the chymotryptic activity of the 20S proteasome.

For comparison, the percentage of inhibition (\(\%I\)) of the chymotryptic activity of the 20S proteasome was calculated independently using specific activity from the following equation:

\[ \%I = 100 \times \left( 1 - \frac{SpA_I}{SpA_U} \right) \]  

(10)

where \(SpA_I\) is the chymotryptic specific activity of the proteasome in the presence of inhibitor, and \(SpA_U\) is the chymotryptic specific activity of the proteasome in the absence of inhibitor.

In studies using blood samples from subjects treated in vivo with PS-341, a baseline sample (untreated or treated with vehicle) from the same study was used to determine the \(k_c/k_T\) with \(\alpha_T = 1.28\), as determined empirically with human blood samples, unless otherwise indicated.