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Assays for Proteasome Inhibition

Peter J. Elliott, Teresa A. Soucy, Christine S. Pien, Julian Adams, and Eric S. Lightcap

1. Introduction

The ubiquitin-proteasome pathway has an essential role in the regulation of numerous cellular proteins, including those mediating inflammatory conditions and cancer ***(1–6)***. Intracellular proteins 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 (MW=2000 kDa) found in high concentration in all mammalian cells. The ATP hydrolytic activity and the specific subunits that bind ubiquitin in the 26S are located within a protein complex known as the 19S subunit which caps either end of the 20S core. The ATP-independent proteolytic activity of the proteasome is contained within this central 20S core (MW = 730 kDa), a multicatalytic protease that has three well characterized peptidase activities. The three peptidases: chymotryptic, tryptic, and postglutamyl peptide hydrolytic activities, are associated with three distinct subunits: $\beta5^*$, $\beta2^*$, and $\beta1^*$, respectively ***(8)***. Each site is defined by its ability to hydrolyze peptide substrates in vitro, with hydrophobic, basic or acidic amino acids in the P_1 position.

Multiple inhibitors of the proteasome have been designed based upon either the natural product, lactacystin ***(9)***, or synthetic peptidyl derivatives ***(10)***. PS-341, a synthetic and potent 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.

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PS-341 has shown significant activity in preclinical murine tumor models ***(3)*** where the anti-tumor activity was positively correlated with the degree of proteasome inhibition measured both in white blood cell and tumor biopsy material.

PS-341 is currently under evaluation as an anti-cancer agent in multiple Phase I and Phase II clinical trials in which blood levels of the drug are being measured by LC/MS/MS to determine standard pharmacokinetic parameters. Based on animal models (including nonhuman primates), the drug is rapidly distributed throughout the body and plasma levels fall to near detection limits within minutes of intravenous dosing. The pharmacodynamic profile of the drug has also been evaluated to assess the activity of PS-341 at its target site, the proteasome. As such, it is possible not only to evaluate blood levels of PS-341 but also to record the level of enzyme inhibition over time.

To explore the possibility that the proteasome activity assay could be used in future preclinical studies and in clinical trials, 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 ***(12,13)***. The focus of the method development was to obtain a simple rapid and reproducible assay that could be used to determine accurately the level of proteasome activity in rodent blood samples treated with PS-341. To confirm that the tryptic and chymotryptic activities were only owing to the proteasome, a proteasome inhibitor from a second structurally unrelated class was also employed, *clasto*-lactacystin-β-lactone (lactacystin). Using multiple assays it was possible to cross-validate each format and determine the optimal assay methodology.

Herein, we describe the development of these pharmacodynamic assays to record proteasome activity present within biological material. This unique method of measuring proteasome activity 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 sub-populations of blood cells. As such, this method will allow the determination of the activity of inhibitors at their biological target, the proteasome, and provide a method for studying their pharmacodynamics as an alternative, or an additional procedure, to pharmacokinetic measurements.

In summary, 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 sub-populations of blood cells.

Currently, the assays are being explored in clinical trials to validate the methods and to determine the optimum conditions for collection, storage and preparation of samples. To date, the assays provide a source of real-time pharmacodynamics on individual patients dosed with PS-341. In the future, these results will be invaluable for correlation with drug blood levels, safety and clinical activity data. In addition, the resolution of issues around measuring blood cell proteasome inhibition should be applicable to other biological material (e.g., tissue biopsy). Finally, the current cuvet-based version of the assay should be readily adaptable to a high-throughput 96-well plate format.

2. Materials

Chemicals should be the purest grade commercially available. All aqueous solutions should be prepared with water purified by reverse osmosis or ion exchange further treated with a Millipore MilliQ Plus UF water purifying system (or equivalent system) resulting in water with a resistivity greater than 16 MΩ•cm.

1. Pierce Coomassie Plus Protein Assay kit (or equivalent).
2. Phosphate buffered saline (PBS): 10 m*M* phosphate, 2.7 m*M* KCl, 137 m*M* NaCl, pH 7.4 ± 0.1.
3. 1 *M* HEPES, pH 8.2 ± 0.1.
4. 0.5 *M* ethylene diamine tetraacetic acid (EDTA), pH 8.0 ± 0.1.
5. Chymotryptic substrate (Bachem): Dissolve Suc-LLVY-AMC in DMSO (25 mg/5.45 mL) and store resultant 6 m*M* substrate in 100 µL aliquots at –20°C (–25 to –10°C).
6. Tryptic substrate (Bachem): Dissolve Bz-VGR-AMC in DMSO (50 mg/8.46 mL) and store resultant 10 m*M* substrate in 100 µL aliquots at –20°C (–25 to –10°C).
7. 2% sodium dodecyl sulfate (SDS): Prepare 2% SDS in water (2 g/100 mL) in a glass bottle. Store in 1 mL aliquots at –20°C (–25 to –10°C). Do not use Corning polystyrene filter system flasks for SDS preparation or storage.
8. AMC Stock Solution: AMC is dried under vacuum for 3 d, then dissolved in DMF (0.114 mg/mL). Store the 20 m*M* AMC stock solution in 1 mL aliquots at –20°C (–25 to –10°C) in screw-top vials.
9. Blood collection tubes containing sodium heparin.
10. Becton Dickinson Vacutainer CPT Cell Preparation Tubes.
11. Microcentrifuge capable of 6600*g*.
12. Microfuge tubes (1.5 mL).
13. Conical centrifuge tubes (15 and 50 mL).
14. Visible range 96-well microplate reader.
15. Fluorometer (e.g., Hitachi F-4500 fluorometer) with temperature control.
16. 3-mL disposable polystyrene fluorescence cuvets.
17. Magnetic cuvet stir bars.
18. Chymotryptic Substrate Buffer: 20 m*M* HEPES, pH 8.2, 0.5 m*M* EDTA, 0.05% SDS, 1% DMSO, 60 µ*M* LLVY-AMC. To a clean 50 mL glass bottle (rinsed with

methanol and air-dried), add 600 µL 1 *M* HEPES, pH 8.2, 30 µL 0.5 *M* EDTA, pH 8.0, 750 µL 2% SDS, 300 µL 6 m*M* LLVY-AMC, and bring to volume (30 mL) with water. Chymotryptic substrate buffer (30 mL; typical batch) is prepared and can be stored at 4°C for up to 1 mo. This is sufficient for testing 15 samples (2 mL/sample). Larger batches may be prepared if stored at 4°C. Only the amount of chymotryptic substrate buffer to be used within 24 h should be brought to 37°C ± 2°C.

19. Chymotryptic Substrate Buffer for maximal activity from white blood cells: (20 m*M* HEPES, 0.5 m*M* EDTA, 0.035% SDS, 1% DMSO, 60 µ*M* LLVY-AMC). To a clean 50 mL glass bottle (rinsed with methanol and air-dried), add 600 µL 1 *M* HEPES, pH 8.2, 30 µL 0.5 *M* EDTA, pH 8.0, 525 µL 2% SDS, 300 µL 6 m*M* LLVY-AMC, and bring to volume (30 mL) with water. Proteasome activity from white blood cells can be measured accurately using the assay buffer listed in **step 18**, but this assay buffer will give better activity with white blood cell samples of relatively low activity. To determine inhibition by specific activity, samples must be compared using the same assay buffer. Whole blood cell samples require 0.05% SDS to give accurate results.
20. Tryptic Substrate Buffer (20 m*M* HEPES, 0.5 m*M* EDTA, 0.6% DMSO and 60 µ*M* VGR-AMC). To a clean 50 mL glass bottle (rinsed with methanol and air-dried), add 600 µL 1 *M* HEPES, pH 8.2, 30 µL 0.5 *M* EDTA, pH 8.0, 180 µL 10 m*M* VGR-AMC, and bring to volume (30 mL) with water. Tryptic substrate buffer (30 mL; typical batch) is prepared and can be stored at 4°C for up to 1 mo. This is sufficient for testing 15 samples (2 mL/sample). Larger batches may be prepared if stored at 4°C. Only the amount of tryptic substrate buffer to be used within 24 h should be brought to 37°C ± 2°C.

3. Methods

This protocol describes the ex vivo assay method for measurement of proteasome activity in peripheral whole blood or white blood cells. The assay is based upon the chymotryptic and the tryptic activities of the proteasome. It uses fluorometry to measure the rate at which the proteasome hydrolyzes an amide bond in a small peptide substrate. Measurement of these rates in the absence and presence of an inhibitor allows a determination of what proportion of the proteasome is bound by an inhibitor. For tightly bound inhibitors, this assay can be correlated with the amount of inhibition of the proteasome in vivo. In addition, since some inhibitors of proteasome activity completely inhibit the chymotryptic activity but do not inhibit the tryptic activity, the percent of proteasome bound by such an inhibitor can be directly determined by the ratio of the chymotryptic and tryptic activities. This assay has been used to measure proteasome activity in peripheral whole blood or white blood cells in humans, Cynomolgus monkeys, dogs, rats, and mice. There are several steps involved in the measurement of 20S proteasome activity in blood cells. These include preparation of the blood sample, protein assay of the sample, fluorometer

calibration, fluorometric assay of both chymotryptic and tryptic activities, and calculation of results.

3.1. Preparation of Peripheral Whole Blood (PWB) Sample

1. Collect the required amount of blood into a tube containing anticoagulant (e.g., heparin).
2. The blood is then treated using the sample transfer protocols (*see* **Notes** for other protocols).
3. Sample Transfer Protocol: The cells from the collected blood (PWB) may be prepared as follows: (a) Transfer whole blood (10–1000 µL) to a 1.5 mL microfuge tube; (b) Centrifuge at 6600*g* for approx 10 min at 4 ± 3°C; (c) Aspirate off supernatant; (d) Pellet may be frozen at –70 ± 10°C or on dry ice. These frozen pellets may be shipped to the site of analysis on dry ice. Pellets should stored at –70 ± 10°C for no more than 2 yr.
4. Whole Blood Lysates are prepared by the following procedure: (a) If samples are frozen, quick thaw samples by placing them in a 37 ± 2°C bath for a couple of minutes; (b) Place blood samples on ice; (c) Transfer 10 µL sample to a clean 1.5-mL microfuge tube; (d) Lyse the cells by adding 300 µL 5 m*M* EDTA to each sample (0.5 mL 0.5 *M* EDTA in 50 mL water). Let the samples stand on ice at least 15 min. Red blood cells that have been frozen will be substantially lysed but all procedures should still be followed; (e) Centrifuge at 6600*g* for approx 10 min at 4 ± 3°C; (f) Carefully collect the supernatant (~250 µL) into a clean 1.5 mL microfuge tube; (g) Add an equal volume of 40 m*M* HEPES, 1.0 m*M* EDTA, 20% glycerol, pH 7.6 to the supernatant. Mix by tapping the microfuge tube; (h) Store the lysate sample frozen at –70 ± 10°C for no more than 2 yr. Lysate samples may be shipped on dry ice.
5. Samples may be thawed and refrozen up to 10×.

3.2. Preparation of Peripheral White Blood Cell (WBC) Samples

Peripheral white blood cells are separated from blood samples upon collection and lysed for storage at –70°C until tested. To prevent interference with the assay it is important that the sample preparation remove all red blood cells. If the sample is contaminated with red blood cells, chymotryptic assays should be done at 0.05% SDS. Separation is done in Becton Dickinson Vacutainer CPT Cell Preparation Tubes.

1. Draw 8 mL of blood into CPT tubes and invert 5×, gently.
2. Within 10 min of blood draw, spin for 30 min at 1500*g* at 25°C.
3. Discard the upper half of the plasma layer.
4. Transfer the remaining plasma layer above the plug to a 15-mL polypropylene conical tube. Be careful not to include any red blood cells (RBCs). If RBCs are inadvertently collected, put all plasma back into CPT tubes and respin.
5. Wash with PBS and respin at 600*g* for 10 min at 25°C.

6. Carefully pour off the supernatant and allow the tubes to drain for 3 min. A cotton swab may be used to remove any other drops that may have remained in the tubes.
7. Store WBC pellets at –70°C until ready for lysis or shipment on dry ice.
8. Pour off the supernatant and resuspend the pellet in ~1 mL cold PBS.
9. Transfer the suspension to a 1.5-mL microfuge tube.
10. Centrifuge at 6600*g* for 10 min at 4°C.
11. Aspirate off the supernatant.
12. Lyse the cells by adding 200 µL 0.5 m*M* EDTA to each sample. Let the samples stand on ice for at least 15 min.
13. Centrifuge at 6600*g* for 10 min at 4°C.
14. Carefully collect the supernatant (~200 µL) into a clean 1.5 mL microfuge tube.
15. Store lysate sample frozen at –70 ± 10°C.

3.3. Coomassie Protein Assay

1. Perform the Coomassie (Bradford) protein assay in duplicate in accordance with manufacturer's instructions.
2. Dilute the BSA protein standard (2 mg/mL) 1 : 1 with water. This 1000 µg/mL stock solution is further diluted to 500, 250, 125, and 62.5 µg/mL. These standards may be stored at –20°C (–10 to –25°C) and reused.
3. Transfer 10 µL of each standard dilution to wells in duplicate.
4. Transfer 10 µL of sample supernatant into a 96-well microplate with 90 µL water (1 : 10 dilution).
5. Add 10 µL of the 1 : 10 diluted sample to new wells in duplicate.
6. Add 300 µL Coomassie Plus to wells containing standards and diluted samples.
7. Read on the microplate reader at 595 nm.
8. Prepare a standard curve and calculate sample concentrations.
9. The duplicate protein results must differ by no more than 10%, otherwise repeat the assay.

3.4. Fluorometer Calibration

1. Thaw a vial containing 20 m*M* AMC stock solution and prepare a 50 µ*M* AMC solution (5 µL 20 m*M* AMC stock solution into 2 mL DMF). Refreeze 20 m*M* AMC stock solution.
2. Prepare 2 µ*M* AMC by 1 : 25 dilution of 50 µ*M* AMC stock solution in DMSO.
3. Record the zero value for chymotryptic substrate buffer under assay settings (λ_{ex} = 380 nm; λ_{em} = 440 nm; excitation band width = 10 nm; emission band width = 20 nm). Add 5 µL of 2 µ*M* AMC into 2 mL of chymotryptic substrate buffer approx every 30 s for a total of five times to produce a calibration curve for 0–50 pmol AMC. Record the fluorometer calibration values.

3.5. Fluorometric 20S Proteasome Assay

1. Make sure that the chymotryptic or tryptic substrate buffer has equilibrated to 37 ± 2°C before running samples.

2. Rinse a magnetic cuvette stir bar and 3 mL polystyrene fluorometric cuvet with deionized water and methanol.
3. Place the magnetic cuvette stir bar in the 3 mL polystyrene fluorometric cuvet.
4. Add 2 mL of chymotryptic or tryptic substrate buffer (at 37 ± 2°C) to the cuvet.
5. Check that the baseline is stable with minimal noise (*see* **Notes 3–5**).
6. Add 10–100 µg of test sample to the cuvette and let the reaction run for 5–10 min (λ_{ex} = 380 nm; λ_{em} = 440 nm).
7. Measure the maximum linear slope (for at least 1 min of data, *see* **Note 7**).
8. If the rate is less than 1 pmol AMC/min, repeat the measurement using twice the amount of test sample used in **step 6**.

3.6. Calculations

1. Specific Activity:

$$SpA_C = \{(m_C)/(0.000001 \times vol_C \times [pn])\}/m_{fluor})/60$$

where SpA_C is the chymotryptic specific activity of the blood sample (pmol AMC/s•mg protein), m_C is the slope from the chymotryptic assay (FU/min), vol_C is the volume of blood sample added to the chymotryptic assay (µL), [pn] is the concentration of protein in the sample added to the chymotryptic assay (µg/mL), and m_{fluor} is the slope from the fluorometer calibration (FU/pmol AMC).

2. Chymotryptic to tryptic activity ratio:

$$v_C/v_T = (m_C \times vol_T)/(m_T \times vol_C)$$

where v_C/v_T is the ratio of the chymotryptic activity to the tryptic activity in a blood sample, m_C is the slope from the chymotryptic assay (FU/min), vol_T is the volume of blood sample added to the tryptic assay (µL), m_T is the slope from the tryptic assay (FU/min), and vol_C is the volume of blood sample added to the chymotryptic assay (µL).

3. Percent inhibition by specific activity:

$$\%I(SpA) = 100 \times (1 - SpA_{Ci}/SpA_{Cu})$$

where %I(SpA) is the percent inhibition of chymotryptic proteasome activity calculated using specific activity, SpA_{Ci} is the specific activity from the chymotryptic assay in the inhibited sample, and SpA_{Cu} is the specific activity from the chymotryptic assay in the uninhibited sample.

4. Percent inhibition by chymotryptic to tryptic ratio:

$$\%I(C:T) = 100 \times (k_C/k_T - v_C/v_T)/(k_C/k_T - v_C/v_T + 1.35 \times v_C/v_T)$$

where%I(C:T) is the percent inhibition of the chymotryptic proteasome activity calculated using the ratio of chymotryptic to tryptic proteasome activities from a blood sample, k_C/k_T is the ratio of chymotryptic to tryptic activities in the uninhibited blood sample, and v_C/v_T is the ratio of chymotryptic to tryptic activities in the inhibited sample.

3.7. Assay Validity

The following assay results are typical of clinical results for whole blood assays evaluated thus far. Although significant variation occurs in values from multiple individuals, variation within an individual is not a problem.

1. The sample protein concentration is 500–6000 µg/mL.
2. The ratio of the chymotryptic to tryptic activities (in pmol AMC/s × mg protein) prior to treatment is 0.9–3.5.
3. The chymotryptic specific activity prior to treatment is 0.4–1.3 pmol AMC/s × mg protein.
4. The tryptic specific activity before or after treatment is 0.2–0.8 pmol AMC/s × mg protein.
5. The chymotryptic specific activity for white blood cells prior to treatment is 25–35 pmol AMC/s × mg protein.

4. Notes

4.1. Sample Transfer Protocols

1. The collected blood may be frozen on dry ice immediately after collection. These samples should be transferred to the analytical site on dry ice. These samples should be stored at –70 ± 10°C for no more than 2 yr.
2. The collected blood may be stored and shipped on ice. These samples should be received and processed no more than 48 h after collection.

4.2. Fluorometric Assays

3. Dust in the cuvet will create significant noise. Rewash the stir bar and cuvet, if necessary.
4. High noise may result from a fast stir bar.
5. A rolling baseline indicates a stopped stir bar.
6. Complete activation of the 20S proteasome in the presence of SDS is achieved within 10 min. Consistent results for the chymotryptic assay are obtained for readings taken after 4 min and up to 10 min.
7. Activation of the proteasome is not seen in the absence of SDS. Therefore the maximum slope for the tryptic assay may occur anywhere within the 10 min reaction curve. In general, the maximum slope has been observed between 1.5 and 3 min.

4.3. Modifications for 96-Well Plate Format

This assay has been modified for 96-well plate format. A Packard Instruments Multiprobe 104DT workstation was used to format plates and a BioTek FL600 microplate spectrofluorometer was used to follow the kinetics at 37°C (λ_{ex} = 380 nm (20 nm bandwidth), λ_{em} = 450 nm (50 nm bandwidth)).

8. The assay is linear for 1–10 µg protein/well. Dilute the blood sample, if necessary, in 10 m*M* HEPES, 1 m*M* EDTA, 10% glycerol, pH 8.0 to obtain 30–300 µg protein/mL.
9. Change the final volume for both assay buffers from 30 mL to 23 mL (1.3X concentrated) and heat to 37°C.
10. Pipet 30 µL blood sample/well into a black 96-well fluorometric plate (Corning/Costar plate #3915) in triplicate.
11. Pipet 100 µL chymotryptic assay buffer/well and place plate on plate carrier and begin assay. Read the change in fluorescence every 40 s for 25 min with a 4 s shaking at setting 2 between each time the plate is read.
12. The average slope from 8–25 min is determined to calculate the rate in the chymotryptic assay.
13. Pipet 30 µL blood sample (30–300 µg protein/mL)/well into a black 96-well fluorometric plate (Corning/Costar plate #3915) in triplicate.
14. Pipet 100 µL tryptic assay buffer/well and place plate on plate carrier and begin assay. Read the change in fluorescence for 25 min using the same protocol as for the chymotryptic assay.
15. The average slope from 1–18 min is determined to calculate the rate in the tryptic assay.
16. Calculations are done as with the cuvet-based assay.

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