Proteasome proteolytic activity in hematopoietic cells from patients with chronic myeloid leukemia and multiple myeloma

T he proteasome is a multicatalytic complex which acts as the main non-lysosomal proteolytic machinery of the cell. Found in both the nucleus and cytoplasm of all eukaryotic cells, the proteasome is crucial in the control of numerous cell functions. The complex role of the proteasome includes degradation of proteins associated with cell cycle control, apoptosis and angiogenesis.1-4 High levels of proteasome have been demonstrated in leukemic cell lines by immunocytochemistry and inhibition of proteasome activity in some malignant cell types as opposed to in their normal counterparts and first generation compounds are currently in clinical trials for the treatment of multiple myeloma. The objective of our study was to develop a method to extract and measure functional proteasome activity in primary human cells so that this method could then be used to determine whether patients might benefit from proteasome inhibitor therapy.

Results. Cells expressing bcr-abl showed significantly higher proteasome levels (372±16 AFU/1×10^6 cells/min) than did bcr-abl-negative cells (151±8 AFU/1×10^6 cells/min) and were more sensitive to induction of apoptosis by proteasome inhibitor. Human myeloid leukemia cell lines showed higher levels of activity than those representing myeloma (eg HL-60 cells 947±25 AFU/1×10^6 cells/min; U266 177±6 AFU/1×10^6 cells/min). Primary cells from patients had similar levels of activity to those of the comparable cell line model.

Interpretation and Conclusions. This simple method measures functional proteasome activity in primary leukemic cells and demonstrates for the first time that this activity is higher in myeloid leukemia than in myeloma cells.

Key words: proteasome, inhibitors, proteolytic activity, leukemia.
improved method to extract and measure ChT-L activity in hematopoietic cells which could be used to screen potential candidates for proteasome inhibitor therapy and to use the assay to measure functional proteasome activity in other malignant cell types.

**Design and Methods**

**Cell lines**

FDCP-MIX cells transfected with a temperature-sensitive bcr-abl construct (ts-bcr-abl FDCP-MIX) were a gift from A.D. Whetton, UMIST and were used as a model system for chronic myeloid leukemia (CML). When these cells are grown at the permissive temperature of 32°C the bcr-abl tyrosine kinase is active and the FDCP-MIX cells remain growth factor-independent and retain their ability to differentiate so displaying the characteristics of chronic phase CML cells; when grown at the restrictive temperature (39°C) the bcr-abl kinase is inactive. Mock transfected cells (transfected with vector alone) grown at 32°C were used as an additional control. Cells were maintained in Fischer’s medium (Invitrogen Ltd., Paisley, UK) supplemented with 20% horse serum and 5% murine interleukin-3 at 37°C in a humidified atmosphere of 5% CO₂.

Other cell lines used were purchased either from the European Collection of Cell Cultures (HEI, HL-60, HS-SULTAN, K562, U266) or the Department of Human and Animal Cell Culture, Germany (DSMZ:KG1a, NB4, OPM-2). Cells were maintained in RPMI-1640 plus 10% fetal calf serum (FCS) and penicillin/streptomycin (5,000 units/mL). All culture reagents were obtained from GibcoBRL, Life Technologies, Paisley, UK.

**Primary human cells**

Bone marrow aspirate samples were obtained with informed consent from normal donors and patients at diagnosis or at relapse. Aspirates were collected in RPMI-1640 supplemented with 10% FCS and containing 100 IU preservative-free heparin (Leo Laboratories Ltd., Bucks, UK). Mononuclear cells were separated over Ficoll-Hypaque using standard procedures.

**Extraction of proteasome from cells**

**Extraction with 5% SDS**

Cells were pelleted by centrifugation and lysed with 5% SDS in phosphate-buffered saline (PBS). Cells (1×10⁶) were washed once in PBS by centrifugation at 350 g for 5 minutes at room temperature and the cell pellet lysed with 1 mL of 5% SDS in PBS for 10 minutes on ice. The cells were centrifuged at 350 g for 5 minutes at room temperature, and the resulting supernatant removed for assay.

**Extraction with 0.1% Triton X-100**

Cells were lysed in 0.1% Triton X-100 in PBS and processed as described above for SDS extraction.

**Extraction with ATP/DTT lysis buffer**

Cells were pelleted by centrifugation and resuspended in ATP/DTT lysis buffer (10 mM Tris-HCl pH 7.8, 0.5 mM DTT, 5 mM ATP and 5 mM MgCl₂). Two milliliters of ATP/DTT lysis buffer were used per 2×10⁶ cells. The cell suspension was left on ice for 10 min and then sonicated for 15 s to ensure complete cell lysis. The suspension was then centrifuged at 13,000g for 10 min at 4°C to remove cell debris. The supernatant was collected, 20% (v/v) glycerol was added to the lysates which were stored at -20°C for up to 72 h. Prior to the assay 5 mM EDTA was added to the lysates.

**Determination of proteasome activity**

The fluorogenic substrate N-Succinyl-Leu-Leu-Val-Tyr-AMC (Succ-LLVY-AMC; Sigma-Aldrich, Dorset, UK) was used to measure the main chymotrypsin-like activity of the proteasome. Substrate (2 µL) at a concentration of 10 mM was added to the prepared cell lysates. Proteasome activity was determined as the increase in fluorescence due to turnover of the fluorescent substrate measured for 35 min at one minute intervals; activity was expressed as arbitrary fluorescence units (AU). Fluorescence was measured on a Cytofluor Series 400 (Applied Biosystems, Warrington, UK) using excitation and emission wavelengths of 395±25 and 460±40 nm, respectively.

**Apoptosis assays**

The ability of the proteasome inhibitor BzLLLCOCHO to induce apoptosis in the ts-bcr-abl FDCP-MIX cell line was assessed by Hoechst 33342/propidium iodide and Mitosensor™ stains. The proteasome inhibitor BzLLLCOCHO was synthesized as previously described and was stored as a 10 mM stock solution in dimethyl sulfoxide (DMSO). Cells (2×10⁶/µL) were cultured with 5 µM BzLLLCOCHO and examined over a period of 72 h to determine their apoptotic status.

The Hoechst 33342/propidium iodide stain was used to assess membrane integrity and nuclear morphology, respectively. Hoechst 33342 stock solution (1 mg/mL in DMSO) was added to approximately 1×10⁶ cells in culture media (1 µL per 200 µL) and the cells incubated at 37°C for 20 minutes. Cells were pelleted and resuspended in 10 µL of 10 µg/mL propidium iodide in PBS. Apoptosis was assessed using a fluorescent microscope with a DAPI band-pass filter at ×40 magnification.

Apoptosis was additionally assessed using the ApoAlert™ Mitochondrial Membrane Sensor Kit (Clon-
tech UK, Hampshire, UK). This kit is based on the collapse in the mitochondrial inner transmembrane potential ($\Delta \Psi_m$) following the induction of apoptosis. This change in $\Delta \Psi_m$ facilitates the release of caspase-activating proteins from the mitochondria into the cytosol. The cationic dye, Mitosensor$^{\text{TM}}$, is taken up by the mitochondria of non-apoptotic cells where it forms aggregates that fluoresce red; due to the altered transmembrane potential apoptotic cells cannot take up the dye and fluoresce green. The kit was used according to the manufacturer’s instructions. Apoptosis was assessed under a fluorescent microscope using a band-pass filter to detect fluorescein and rhodamine, at ×40 magnification.

Results

The ts-bcr-abl FDCP-MIX cell line model was used to develop the proteasome extraction method. This model cell line provides a population of cells that are leukemic (cells grown at the permissive temperature) and their normal counterparts (cells grown at the restrictive temperature), and thus allows an examination of the relationship between proteasome activity and oncogenic transformation.

Extraction of proteasome by conventional methods

Extracts prepared from cells lysed with SDS or Triton X-100 showed no detectable turnover of the substrate. No proteasome activity was detected even when the number of cells extracted was increased from $1 \times 10^6$ to $5 \times 10^6$ and the time course of the measurements was extended to 720 min.

Extraction of proteasome by ATP/DTT lysis buffer

FDCP-MIX cells lysed with the ATP/DTT lysis buffer caused a rapid turnover of the fluorescent substrate. To establish that the activity measured by the increase in fluorescence of the substrate was due to cleavage of the amide bond by the chymotrypsin-like activity of the proteasome, the peptide glyoxal inhibitor BzLLLCOCHO$^{25}$ was added to the ATP/DTT extract at a concentration of 10 $\mu$M and the fluorescence monitored. This peptide is a potent inhibitor of the proteasome and is some 100-fold and 300-fold less potent an inhibitor of the cysteine proteinases calpain and cathepsin B, respectively.$^{25}$ Addition of BzLLLCOCHO caused 90.5% inhibition of cleavage of the fluorogenic substrate by the proteasome extracts prepared with the ATP/DTT lysis buffer (Figure 1).

Stability of proteasome activity post-extraction

The ATP/DTT lysis buffer was found to be a reproducible method for extracting proteasome activity from cells. Proteolytic activity was determined initially in freshly prepared samples. Samples were then stored at −70°C and proteasome activity re-evaluated after storage. Activity of proteasome extracts decreased significantly after 24 h storage and was undetectable after 48 h. It was initially attempted to stabilize the proteasome extract by using commercially available protease inhibitors directed against the four main classes of protease found in eukaryotic cells (Table 1).

The recommended working concentrations of each protease inhibitor were added individually to the proteasome extracts and the activity of the proteasome measured by the release of the fluorophore from the peptide substrate. The activity in each sample, as well as in an untreated sample as the control, was measured at 0, 24 and 48 h time points. By 24 h post-extraction the untreated (no inhibitor) sample showed no turnover of fluorescent substrate. Pefabloc and EDTA were the only two inhibitors that maintained some substrate turnover, albeit at a very low rate. Optimum substrate turnover was maintained by the addition of 5 mM EDTA in combination with 20% glycerol.

Table 1. Protease inhibitors evaluated for stabilization of proteasome extracts.

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<thead>
<tr>
<th>Protease class</th>
<th>Specific protease inhibitor</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Serine proteases</td>
<td>Pefabloc</td>
<td>1 $\mu$M</td>
</tr>
<tr>
<td>Cysteine proteases</td>
<td>E-64</td>
<td>10 $\mu$M</td>
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<tr>
<td>Metallo-proteases</td>
<td>EDTA</td>
<td>1 $\mu$M</td>
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<tr>
<td>Aspartate proteases</td>
<td>Pepstatin</td>
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Proteasome activity in myeloid leukemia cell lines

Proteasome activity was measured in the murine ts-bcr-abl-FDCP-MIX cell line model with cells cultured...
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at permissive and restrictive temperatures to test the hypothesis that leukemic cells exhibit higher proteasome activity than do non-leukemic cells. 

bcr-abl-positive cells displayed significantly higher turnover of the fluorescent substrate than did bcr-abl negative cells (Figure 2). Addition of BzLLLCOCHO (10 µM) decreased the fluorescence by 90.5% confirming that cleavage of the peptide substrate was due largely to an active 26S proteasome. This demonstrates that cells mimicking the leukemic phenotype have higher active proteasome levels than do cells with the normal, non-leukemic phenotype. Furthermore bcr-abl-positive cells were more sensitive than bcr-abl- negative cells to induction of apoptosis by BzLLLCOCHO, suggesting that measurement of functional proteasome activity may provide an indication of sensitivity to proteasome inhibition (Figure 3). Proteasome activity was also measured in five human myeloid leukemia cell lines: KG1a, HL-60, NB4, HEL and K562. All five cell lines showed high levels of proteasome activity with NB4 exhibiting the highest and KG1a the lowest activities (Figure 4). This confirms that elevated proteasome levels are a common feature of leukemic cells.

Proteasome activity in multiple myeloma cell lines

PS-341 is a commercially available potent and specific inhibitor of the proteasome. It has demonstrated anti-tumor activity against multiple myeloma cells in vitro, both when used alone and when used in combination with other agents. Since PS-341 is currently in clinical trials for the treatment of myeloma, proteasome activity levels were investigated in multiple myeloma. Proteasome activity was measured in two standard human multiple myeloma cell lines, U266B1 and OPM-2. Activity was also measured in the HS-Sultan cell line which was originally thought to be a multiple myeloma line but was subsequently shown to be a subclone of the Burkitt’s lymphoma-derived cell line Jijoye.

Similar proteasome levels were found in the two myeloma cell lines (177±6 and 169.3±2.9 AU/1×10⁶ cells/minute for the U266B1 and OPM-2 lines, respectively) whilst the Burkitt’s lymphoma line had significantly lower activity (87.7±4.2 AU/1×10⁶ cells/minute) (Figure 5).

Proteasome activity in primary human cells

Bone marrow samples were obtained from patients with CML or multiple myeloma at diagnosis or in relapse. After Ficoll–Paque separation, proteasome was extracted from 1×10⁶ cells from each patient’s sample using the ATP/DTT lysis buffer. The activity of the proteasome was measured by monitoring release of the fluorophore from the peptide substrate Succ-LLVY-AMC. Proteasome activity was significantly higher in cells from all patients studied than in normal marrow cells (Figure 6). Activity in primary cells was comparable to that observed in the equivalent cell line. In CML
patients the range of proteasome activity was 360 to 1220 AFU/1×10^6 cells/minute, with a median value of 485 AFU/1×10^6 cells/minute. This compares favorably with the proteasome activity of 372 AFU/1×10^6 cells/minute measured in the ts-bcr-abl-FDCP-MIX cell line model. Similarly, patients with myeloma had a median protease level of 158 AFU/1×10^6 cells/min which is comparable to the activity of 169.3±2.9 AFU/1×10^6 cells/minute observed in the myeloma cell line OPM-2. Proteasome levels in both primary CML cells and cell lines were higher than in myeloma.

Discussion

The proteasome is now recognized as a novel therapeutic target and first generation proteasome inhibitors are currently in phase II and III clinical trials for multiple myeloma and a range of solid tumors. Proteasome inhibitors induce apoptosis in leukemic cell lines in vitro but little is known about functional proteasome activity in primary leukemic cells. A recent phase I study of PS-341 in refractory or relapsed acute leukemia demonstrated evidence of transient hematologic improvements in some patients. Although the study was small, the authors felt further investigation of proteasome inhibitors was warranted in this clinical setting. We have developed a method to measure proteolytic activity in hematopoietic cells which is suitable for screening peripheral blood or bone marrow samples from patients. This may allow us to predict which patients might benefit from proteasome inhibitor therapy when it becomes more widely available.

Immunocytochemical studies originally demonstrated that proteasome levels were higher in leukemic cell lines than in normal mononuclear cells and that proteasome expression decreased as leukemic cells were induced to differentiate. More recently, ELISA assays have been used to detect the proteasome in plasma from patients with both solid tumors and hematopoietic malignancies. These have shown no correlation between proteasome level and disease activity in hematologic disorders and a very modest association in solid tumors. This is unsurprising given that the proteasome is located within the nucleus and cytoplasm of the cell and plasma levels may simply reflect tumor cell lysis.

Lightcap and colleagues developed a pharmacodynamic assay to facilitate clinical studies with the proteasome inhibitor PS-341. This assay has been used to calculate both the chymotrypsin to trypsin ratio and the percentage of proteasome inhibition in whole blood from patients with tumors receiving treatment with PS-341. The percentage of proteasome inhibition is derived from measurements of chymotrypsin-like activity with baseline values for each individual patient set as 100%. This allows the change in blood proteasome activity due to inhibitor therapy to be monitored; the ability of PS-341 to reach and inhibit tumor proteasome activity may be different. The disadvantage of this approach is that it does not allow simple direct comparisons of absolute proteasome activity in tumors either between patients or between different disease states. Our knowledge of the proteolytic activity of the proteasome is mainly based on pure extracts derived from cell lines. These studies use large numbers of cells and involve several complex purification steps making them unsuitable for clinical investigations. Other investigators have incubated intact cells with similar fluorogenic substrates which makes it difficult to compare different cell types within the same study. We chose to extract proteasome from the cells prior to the assay to minimize the contribution of other intracellular chymotrypsin-like activity and the effects of differential permeability of the substrate.
Kinetic studies have clearly shown that degradation by chymotrypsin is the rate-limiting step in hydrolysis by the proteasome. Proteasome inhibitors currently in clinical trial have been designed to target this component of the enzyme complex specifically. The aim of this study was to develop a simple, robust assay for the chymotryptic activity of the proteasome which could be applied to clinical samples. We have shown in the ts-bcr-abl-FDCP-MIX cell line model that proteasome activity is higher in bcr-abl-positive cells than in bcr-abl-negative cells and that increased proteasome activity is associated with increased sensitivity to induction of apoptosis by proteasome inhibition. Proteasome activity was high in myeloid leukemia as well as in myeloma suggesting that inhibition of the multi-catalytic proteasome complex may be a potential therapeutic strategy for this group of diseases.

LM carried out the majority of the practical work and contributed to data analysis and interpretation. JL made a substantial contribution to designing the procedures for extraction, stabilization and measurement of protease activity and interpretation of data. TCMC was responsible for providing access, information and interpretation regarding clinical samples. BW made a major contribution to the overall conception and design of the project. AEI was responsible for the overall conception and design of the project, data handling and writing most of the manuscript. All authors (with exception of Lynas, deceased) gave their approval of the version submitted for publication. The authors reported no potential conflicts of interest. This paper is not currently being submitted elsewhere.


References


