NEW BIOLUMINESCENT CELL-BASED ASSAYS TO MEASURE ALL THREE PROTEASOME PROTEASE ACTIVITIES

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Here we describe two new cell-based assays for the trypsin-like and caspase-like protease activities of the proteasome. These "add-mix-measure" luminescent assays are sensitive enough to measure proteasome activity directly in multiwell plates.

Introduction

With the success of Millennium Pharmaceutical's dipeptide boronate bortezomib (PS-341) as the first proteasome inhibitor approved by the FDA for the treatment of relapsed refractory multiple myeloma, there remains a strong and continued interest in developing additional proteasome inhibitors that are better tolerated in myeloma treatment and possibly show efficacy in solid tumor cancers (1,2). Several other pharmaceutical companies have proteasome inhibitors in clinical trials, including Proteolix (PR-171 or carfilzomib in phase I and II), Nereus (NPI-0052 or salinosporamide A in phase I) and Cephalon (CEP-18770 in phase I), with second- and third-generation derivatives working their way toward investigational new drug status (3–5).

The 26S proteasome complex contains a core 20S particle, a barrel-like structure that possesses two outer heptameric rings of α subunits and two inner heptameric rings of β subunits. Three major ATPase-independent proteolytic activities, chymotrypsin-like, trypsin-like and caspase-like, are mediated by paired β5, β2 and β1 subunits, respectively, within the constitutive proteasome. Each subunit preferentially cleaves after hydrophobic (β5), basic (β2) or acidic (β1) residues to yield short peptides (3–25 amino acids in length) that are further rapidly degraded upon release from the proteasome (6,7).

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Each of the drugs mentioned above effectively inhibit different proteolytic sites. What remains a consistent theme in current proteasome inhibitor research is that all three sites, not just the chymotrypsin-like, are important in protein degradation (8,9). For example, bortezomib is a covalent but slowly reversible inhibitor that primarily targets β5 and β1 activities; PR-171 preferentially inhibits the β5 activity, and NPI-0052 preferentially inhibits β5 and β2 subunits. During proteasome inhibitor screening, analogs are assessed for numerous properties including mechanism and inhibitory potencies for each of the three major protease activities (β5, β2 and β1). However there is growing interest in how proteasome inhibitors affect the immunoproteasome, which is expressed in hematopoietic cells and antigen presenting cells upon exposure to IFN-γ. The immunoproteasome expresses different β-type subunits, termed LMP7, MECL1 and LMP2 that replace the β5, β2 and β1 subunits, respectively. The immunoproteasome is involved in class I antigen processing (10). Thus, a better understanding of an inhibitor's selectivity and profile for each site ultimately allows compounds with the desired properties (i.e., selective cytotoxicity) to be chosen and aids development of dosing protocols to minimize negative side effects.

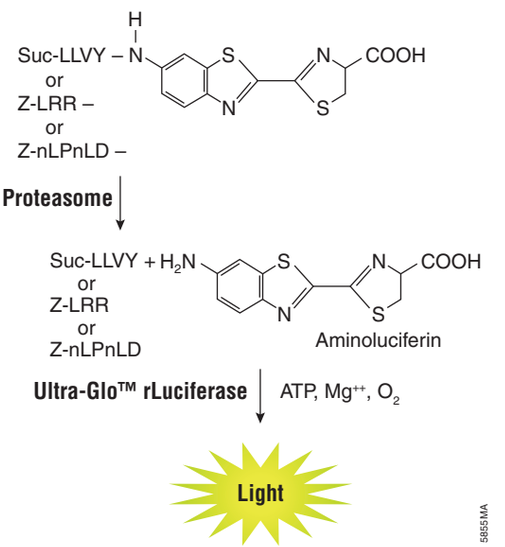

Figure 1. Luminogenic substrates containing either Suc-LLVY, Z-LRR or Z-nLPnLD peptide sequences are recognized by the proteasome. Following cleavage of the substrate by the proteasome, aminoluciferin is released and serves as the substrate for firefly luciferase. Light is produced as a result of the luciferase reaction.

Here we describe two new cell-based assays that allow researchers to measure individually trypsin-like and caspase-like activities of the proteasome in addition to our currently available assay that measures chymotrypsin-like activity. All three assays contain a luminogenic proteasome substrate (Suc-LLVY for chymotrypsin-like, Z-LRR for trypsin-like or Z-nLPnLD for caspase-like measurements) in a buffer optimized for cell permeabilization, proteasome activity and luciferase activity.

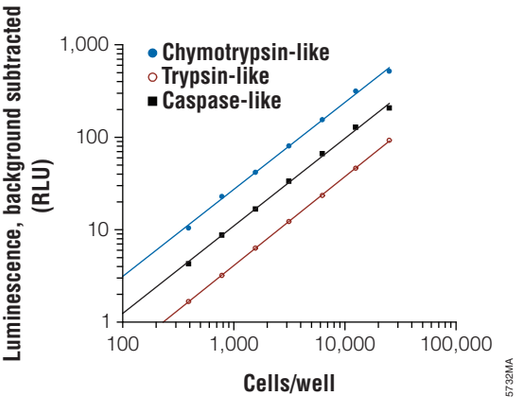

Figure 2. Luminescence is proportional to cell number. U266 cells (human plasma myeloma) were serially diluted in RPMI 1640 medium containing 10% FBS and 1 mM sodium pyruvate as 100 µl/well samples in 96-well plates. Cells then were allowed to equilibrate in a humidified 22 °C, 5% CO_2 incubator for 1.5 hours. Proteasome-Glo™ Cell-Based Assay Reagents each were prepared and equilibrated at 22 °C for 30 minutes before use, during which time the assay plate also was equilibrated. Ten minutes after adding the reagent, luminescence was determined as relative light units (RLU) using a DYNEX MLX® plate luminometer. Each point represents the average of four wells. The results were linear for each assay used (chymotrypsin-like $r^2 = 0.99$, slope = 0.94; trypsin-like $r^2 = 0.99$, slope = 0.96; caspase-like $r^2 = 0.99$, slope = 0.94). The background (no-cell control) was subtracted from each (average no-cell RLU = 3.09 for chymotrypsin, 6.54 for trypsin, 5.39 for caspase). The values for r^2 and slope were calculated after transforming the data to a $\log_{10}$-$\log_{10}$ plot.

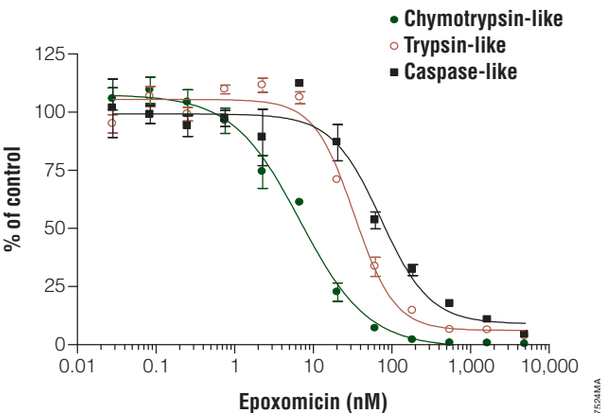

Figure 3. Epoxomicin inhibition of all sites in 384-well format. U266 cells (5,000 cells/well) were plated in 20 µl/well volumes in a 384-well plate. Cells were then equilibrated at 22 °C, 5% CO_2 for 2.5 hours. Serial dilutions of epoxomicin were prepared in culture medium, and 5 µl of each dilution was added to wells. The cells were incubated with the drug for 2 hours at 22 °C, 5% CO_2 before 25 µl/well of each Proteasome-Glo™ Cell-Based Reagent was added. Luminescence was measured with a BMG Lab Technologies FLUOstar Optima luminometer after 15 minutes. The resulting signals were normalized to the untreated controls and plotted using GraphPad Prism® software.

Adding a single reagent directly to cultured cells allows proteasome cleavage of a particular substrate and generates luminescence in a coupled-enzyme format (Figure 1). This format results in luminescence proportional to the amount of proteasome activity in cells as luciferase simultaneously consumes the released aminoluciferin (Figure 2).

Cell-Based Proteasome Measurements in 96- and 384-Well Plate Formats

Our bioluminescent method allows rapid and convenient measurement of proteasome activity using cells in multiwell plate formats without having to prepare cell extracts, which is required for traditional fluorescent proteasome assays. Eliminating the tasks required for each extract generation (data point) allows simple and rapid determination of EC_{50} values for each of the protease activities using either suspension or attached cell types. The sensitivity achieved with our luminogenic proteasome substrates (11) has enabled the development of a direct "add-mix-measure" assay sensitive enough to measure proteasome activity directly in cells in multiwell plates. Figure 3 illustrates epoxomicin inhibition curves for chymotrypsin-, trypsin- and caspase-like activities using a multiple myeloma cell line in a 384-well plate format. Two-hour treatments with epoxomicin, a highly specific and potent irreversible inhibitor of the proteasome, gave EC_{50} values of 7 nM for chymotrypsin-like, 33 nM for trypsin-like and 73 nM for the caspase-like activity.

Specific Trypsin-Like Measurement of the Proteasome

We observed significant nonspecific LRRase protease activity in the serum used to supplement culture medium. Also, background cellular protease activity not associated with the proteasome hindered the specificity of the assay. To minimize these background activities, the trypsin-like assay incorporates a cocktail of protease inhibitors as part of the reagent. These inhibitors were chosen to effectively reduce background without significantly affecting proteasome activity. They are conveniently incorporated into the reagent during its preparation and rapidly inhibit the nonspecific proteases, preventing them from cleaving the luminogenic substrate. As shown in Figure 4, results using epoxomicin-treated H929 cells demonstrate that reagent containing these protease inhibitors significantly reduces both sources of background luminescence, improving assay specificity.

Summary

The Proteasome-Glo™ Cell-Based Assays[a,b] are single-addition, "add-mix-read" luminescent methods that individually measure the chymotrypsin-like, trypsin-like or caspase-like protease activities of the proteasome. In a homogeneous coupled-enzyme format, cleavage of peptide-aminoluciferin substrates releases aminoluciferin, which is used by recombinant luciferase to generate light proportional to the proteasomal activity of the sample. The reagent gently

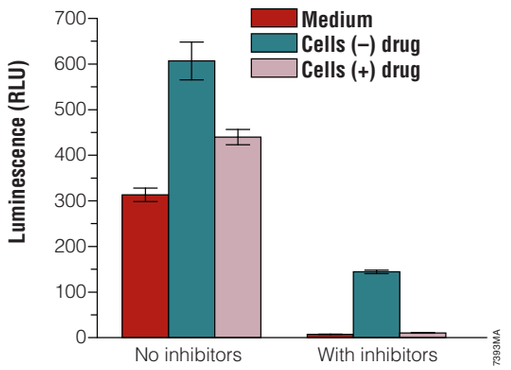

Figure 4. Effectiveness of the protease inhibitors at reducing background. A total of 15,000 H929 cells/well (human plasma myeloma cultured in RPMI 1640 medium containing 10% FBS and 1mM sodium pyruvate) or medium alone were plated in 90 µl/well in a 96-well plate. Cells were cultured overnight at 37 °C, 5% CO_2 before adding 0 or 5 µM epoxomicin (as 10 µl/well additions) for 2 hours. Proteasome-Glo™ Trypsin-Like Reagent was prepared with and without the protease inhibitors, and following a 30-minute equilibration at 22 °C, 100 µl/well was added. Five minutes after adding Reagent, luminescence was determined using a DYNEX MLX® plate luminometer. Reagent containing the protease inhibitors reduced nonspecific LRRase background originating from the serum by 97%. Nonspecific cellular background (as defined by the epoxomicin treatment following medium subtraction) was reduced from 43% to 2%.

permeabilizes cellular membranes, allowing substrate access to the cytosolic proteasome, and eliminates the need to generate cellular extracts. The trypsin-like assay incorporates a combination of protease inhibitors to reduce nonspecific background protease activities originating from both serum and cells. Each of the assays enables and facilitates cell-based measurements of proteasome activity in 96- and 384-well plate formats.

In the Literature: Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay

Groll, M. *et al.* (2008) A plant pathogen virulence factor inhibits the eukaryotic proteasome by a novel mechanism. *Nature*. **452**, 755–8.

The authors of this study investigated the mechanism of action of syringolin A (SylA), which is secreted by virulent strains of the plant pathogen *Pseudomonas syringae*. They show that SylA inhibits all three activities of the proteasome in vitro. They also used the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay to show that SylA inhibits the chymotrypsin-like activity of the proteasome in SK-N-HS neuroblastoma cells.

Filimonenko, M. *et al.* (2007) Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. *J. Cell Biol.* **179**, 485–500.

Endosomal sorting complexes required for transport (ESCRTs) are necessary for sorting membrane proteins into the intralumenal vesicles of the multivesicular body for eventual degradation by the lysosome/vacuole. Mutations in at least one subunit of the ESCRTs are associated with frontotemporal dementia and ALS. In this study, the authors demonstrate that ESCRTs are required for autophagy and prevention of protein aggregation. They address the question of whether loss of ESCRTs might interfere with proteasome activity. Using the Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay, they show that proteasome activity is minimally affected in ESCRT-depleted cells.

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Protocols

Proteasome-Glo™ Cell-Based Assays Technical Bulletin #TB346 (www.promega.com/tbs/tb346/tb346.html)

Ordering Information

Product	Size	Cat.#
Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay	10 ml	G8660
Proteasome-Glo™ Trypsin-Like Cell-Based Assay	10 ml	G8760
Proteasome-Glo™ Caspase-Like Cell-Based Assay	10 ml	G8860
Proteasome-Glo™ 3-Substrate Cell-Based Assay System	3 × 10 ml	G1180

For Laboratory Use. Additional Sizes Available.

[a]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312 and 785294 and European Pat. No. 1131441 have been issued to Promega Corporation for thermostable luciferases and methods of production. Other patents are pending.

[b]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.