Measurement of Three Proteasome Proteolytic Activities Using Luminescent Assays

ABSTRACT
The Proteasome-Glo™ 3-Substrate System consists of three homogeneous, bioluminescent assays that measure the three proteolytic activities associated with the proteasome. Three substrates have been synthesized to monitor the chymotrypsin-like, trypsin-like and caspase-like activities associated with the proteasome. The Proteasome-Glo™ Assays are designed for use with multiwell plate formats and are ideal for automated high-throughput screening of proteasome activity and inhibition.

INTRODUCTION
In eukaryotic cells, the turnover of intracellular proteins is mediated mainly by the ubiquitin-proteasome pathway, a non-lysosomal proteolytic pathway. The 26S proteasome is a 2.5MDa multiprotein complex found in both the nucleus and cytosol of all eukaryotic cells. It is comprised of a single 20S core particle and 19S regulatory particles at one or both ends (1,2) and degrades polyubiquitinated proteins in an ATP-dependent manner. The 19S regulatory unit binds and removes the ubiquitin chains from tagged proteins, and ATPases within the regulatory complex appear to unfold protein substrates and translocate them into the 20S core (3–5).

Three major proteolytic activities (chymotrypsin-, trypsin- and caspase-like) are contained within the 20S core linked to discrete sites. Combined, these activities are responsible for much of the protein degradation required for maintenance of cellular homeostasis, including degradation of critical cell-cycle proteins, tumor suppressors, transcription factors, inhibitory proteins and damaged cellular proteins (6,7).

The importance of the proteasome in regulating turnover of proteins involved in cell cycle control, apoptosis, and angiogenesis has led to the recognition of the proteasome as a therapeutic target for cancer (8–11). A first generation proteasome inhibitor, bortezomib (PS-341), is now an approved drug for the treatment of recurring multiple myeloma, and second generation inhibitors are currently being developed (12). The proteasome is also essential for degrading misfolded and aberrant proteins, and impaired proteasome function has been implicated in diseases such as Parkinson’s and Alzheimer’s (13). Since all three proteolytic activities contribute significantly to protein breakdown and the relative importance can vary widely depending on the protein substrate, monitoring the three activities is essential for understanding the true impact of an inhibitor on protein degradation (14). We have developed three bioluminescent assays to individually monitor the activities of the three proteolytic sites of the proteasome using purified proteasome preparations.

PROTEASOME-GLO™ ASSAY PRINCIPLE
The Proteasome-Glo™ 3-Substrate System (a,b,c) (Cat.# G8531, G8532) consists of three homogeneous, bioluminescent assays that measure the three proteolytic activities associated with the proteasome. Peptide-conjugated fluorophores are widely used as substrates for monitoring proteasome activity, but sensitivity of fluorescent assays can be limited for a variety of reasons. We synthesized luminogenic substrates Suc-LL VY-aminoluciferin, Z-LRR-aminoluciferin and Z-nLPnLD-aminoluciferin to monitor the chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome, respectively. Each substrate is added to a buffer system optimized for proteasome activity and luciferase activity to make a Proteasome-Glo™ Reagent for the particular catalytic activity. The individual Proteasome-Glo™ Reagent is added to test samples in an add-mix-measure format, resulting in proteasome-induced cleavage of the target substrate. Substrate cleavage generates a “glow-type” luminescent signal produced by the luciferase reaction (Figure 1). The Proteasome-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to improve performance across a wide range of assay conditions. The homogeneous Proteasome-Glo™ Assays are designed for use with multiwell plate formats, making the assays ideal for automated high-throughput screening (HTS) of proteasome activity and inhibition.

RAPID, SENSITIVE ASSAYS WITH STABLE SIGNAL
In this coupled-enzyme format, the proteasome and luciferase rapidly reach a steady-state, where the rate of proteasome cleavage of the substrate is equal to the rate of luciferase utilization of the released...
Proteasome activity (Figure 3). At steady-state, the light output is proportional to the rate of proteasome cleavage and thus the amount of proteasome activity (Figure 3). Another feature of the homogeneous, bioluminescent format is that any free aminoluciferin that is a by-product of the peptide-conjugating synthesis is removed prior to exposing the proteasome substrate to the test samples. Consequently, the background is very low and the linear dynamic range is large (Figure 3). The broad linear range and stable signal results in increased sensitivity and flexibility for the bioluminescent proteasome assays. A comparison of bioluminescent and fluorescent proteasome assays demonstrated that the bioluminescent assays are significantly more sensitive and have a much lower limit of detection for proteasome activity (Figure 3). SDS, which is often used to activate the proteasome when measuring the chymotrypsin-like activity, improved the signal-to-noise ratio and linearity of the fluorescent assay using Suc-LLVY-AMC, but the sensitivity still did not approach that of the luminescent assay.

**ROBUST, FLEXIBLE AND ACCURATE ASSAYS**

The sensitivity and speed of the assays and the flexibility in read time make the Protease-Glo™ Assays ideal for high-throughput screening of inhibitors. Since compound libraries are frequently stored in dimethyl sulfoxide (DMSO), we determined the effect of DMSO on the assay. At concentrations up to 1% DMSO, there was no effect on the assay (Figure 4). Between 1 and 10% DMSO, there was a slight decrease in signal for the Chymotrypsin-Like and Caspase-Like Protease-Glo™ Assays and a slight increase in the signal for the Trypsin-Like Protease-Glo™ Assay. Even at concentrations as high as 10% DMSO, the signal decreased by only 40% for the Chymotrypsin-Like Assay, decreased by only 15% for the Caspase-Like Assay, and increased by 39% for the Trypsin-Like Assay (Figure 4).
We used the Proteasome-Glo™ 3-Substrate System to test the known, selective proteasome inhibitor, epoxomicin. The epoxomicin inhibits all three catalytic activities irreversibly, with greatest potency for the chymotrypsin-like site, followed by the trypsin-like, and caspase-like sites (Figure 5). The rank order of potency is consistent with previous reports (14, 15). The broad linear range of the assay allows for accurate analysis of inhibitors with a broad variety of potencies.

CONCLUSIONS
The Proteasome-Glo™ 3-Substrate System adds three new assays to the expanding list of Promega bioluminescent protease assays. This system includes individual assays for all three catalytic sites of the proteasome and is ideal for high-throughput screening for inhibitors when using 20S or 26S proteasome preparations. These assays complement our novel Proteasome-Glo™ Cell-Based Assay that monitors chymotrypsin-like activity directly in cultured cells. All three assays are significantly more sensitive than their fluorescent counterparts. The sensitivity of the assays allows one to use less purified proteasome and/or less test compound while still achieving accurate results. The assays are readily scaled to a 384-well format, and the stability of the luminescent signal provides great flexibility in read time. The rapid time to maximum sensitivity can make the assays very fast to perform.

REFERENCES

PROTOCOL

ORDERING INFORMATION
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*The method of recombinant expression of Coleoptero luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.
*US. Pat. No. 6,600,877, Australian Pat. No. 754312 and other patents and patents pending.
*Patent Pending.
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