MONITORING PROTEASOME ACTIVITY WITH A CELL-BASED ASSAY USING A SINGLE-ADDITION LUMINESCENT METHOD

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Here we introduce the Proteasome-Glo™ Cell-Based Assay. This “add-mix-measure” assay measures the chymotrypsin-like protease activity associated with the proteasome in cultured cells.

Introduction

The 26S proteasome is a 2.5mDa multiprotein complex found both in the nucleus and cytosol of all eukaryotic cells and is comprised of a single 20S core particle and capped by 19S regulatory particles at one or both ends (1,2). The barrel-shaped 20S core contains three major proteolytic activities that are classified by the preferred type of amino acid after which they cleave and are routinely described as chymotrypsin-like, trypsin-like and caspase-like or post-glutamyl peptide hydrolyzing. The ubiquitin-proteasome pathway constitutes a major, nonlysosomal means to maintain cellular homeostasis by degrading misfolded or unassembled proteins and contributes to the activation or suppression of many short-lived critical cell-cycle proteins, tumor suppressors, transcription factors and inhibitory proteins (1,3,4). Here we describe the Proteasome-Glo™ Cell-Based Assay, a homogeneous luminescent assay that measures the chymotrypsin-like protease activity associated with the proteasome complex in cultured cells.

The assay eliminates the initial sample processing tasks and allows measurement of cytosolic proteasome activity directly in a multiwell plate.

Assay Attributes

The Proteasome-Glo™ Cell-Based Assay Reagent contains a luminogenic proteasome substrate, Suc-LLVY-aminoluciferin (succinyl-leucine-leucine-valine-tyrosine-aminoluciferin), within a buffer optimized for cell permeabilization, proteasome activity and luciferase activity. An “add-mix-measure” format results in proteasome cleavage of the substrate and generation of a luminescent signal produced by the luciferase reaction (Figure 1). Because of this design, the assay eliminates the initial sample processing tasks (cell harvesting, washing, mechanical extraction, centrifugation and protein determination) associated with fluorescent substrate protocols and allows measurement of cytosolic proteasome activity directly in a multiwell plate.

Bioassays Using Cultured Cells

Proteasome activity and the inhibition of that activity are typically monitored using either commercially available purified 20S proteasome or crude cellular extracts. Preparing cell extracts can be laborious and time-consuming and is not conducive to high-throughput screening.
Luminescent Cell-Based Proteasome Assay

Experiments using cultured cells can give valuable information about permeability, stability, solubility, synergy and potency of a test compound, whereas using purified 20S proteasome preparations for analyzing inhibitors can miss these complex cellular responses. Lactacystin is an example of a highly specific proteasome inhibitor in which cellular glutathione interacts with the active clasto-lactacystin-β-lactone form of the drug (5). The Hsp90 inhibitor 17-AAG, a geldanamycin analog, has been shown in vitro to have synergistic effects on bortezomib (PS-341) activity with multiple myeloma cells (6). The Proteasome-Glo™ Cell-Based Assay overcomes the limitations of using either cell extracts or purified proteasome preparations. The sensitivity achieved with our luminogenic protease substrates (7) has enabled the development of a direct “add-mix-measure” assay sensitive enough to measure the chymotrypsin-like proteasome activity directly in cells in multiwell plates.

The Proteasome-Glo™ Cell-Based Assay Reagent gently permeabilizes the plasma membrane and rapidly allows the Suc-LLVY-aminoluciferin substrate access to the cytosol. The reagent is formulated to minimize disruption of lysosomes and subsequent nonspecific cleavage of the luminogenic substrate by other non-proteasome proteases. We have used the Proteasome-Glo™ Cell-Based Assay to obtain EC50 values and potency rankings for several inhibitors such as lactacystin and epoxomicin in a variety of cell lines (Figure 4). Furthermore, the assay is easily miniaturized to a 384-well plate to give a Z’-factor value of 0.77 using only 1,500 cells per well (Figure 5), further demonstrating the sensitivity and reproducible performance of this method.

Many proliferating cell types eventually undergo caspase-dependent apoptosis when treated with proteasome inhibitors (8). We were interested to see if we could sequentially multiplex proteasome and caspase measurements on the same sample to look at the kinetics of epoxomicin treatment and induction of caspase-3/7. To do this, we used a modified, concentrated Apo-ONE® Caspase-3/7 Reagent, which contains the profluorescent caspase substrate [Z-DEVD]2-Rhodamine 110. As shown in Figure 6, a 1.5-hour pretreatment of H929 plasma myeloma cells with epoxomicin inhibited proteasome activity in a dose-dependent fashion but did not induce caspase-3/7. However, increasing the treatment duration to 4.5 hours slightly lowered the epoxomicin EC50 value from 9.1 to 3.1nM (consistent with the drug being an irreversible inhibitor), but more importantly, was long enough to show reliable induction of caspase-3/7 activity as measured by the fluorescent caspase product.
Luminescent Cell-Based Proteasome Assay

**Figure 4. Bioassays using proteasome inhibitors. Panel A.** Comparison of lactacystin and epoxomicin using U266 cells. U266 cells (human plasma myeloma; 10,000 cells per well) were plated in 90µl per well volumes in a 96-well plate. Cells were then equilibrated at 37°C, 5% CO₂ for two hours. Serial dilutions of lactacystin or epoxomicin were prepared in culture medium, and 10µl of each dilution was added to wells. The cells were incubated with the drugs for 105 minutes at 37°C, 5% CO₂. The plate was allowed to equilibrate to 22°C before 100µl/well of Proteasome-Glo™ Cell-Based Reagent was added. Luminescence was measured with a DYNEX MLX® luminometer 15 minutes after adding reagent. **Panel B.** Inhibition curves using DU145 and H226 cells. DU145 cells (human prostate; 5,000 cells/well) and H226 (human lung; 2,500 cells per well) were plated in 90µl per well volumes in a 96-well plate. Cells were allowed to attach and equilibrate overnight at 37°C, 5% CO₂. Serial dilutions of lactacystin were prepared in culture medium, and 10µl of each dilution added to the wells. Cells were incubated at 37°C, 5% CO₂ for 105 minutes. The plate was removed and allowed to equilibrate to 22°C before 100µl/well of Proteasome-Glo™ Cell-Based Reagent was added. Luminescence was measured as described for Panel A.

**Figure 5. Z’-factor analysis in 384-well format.** Z’-factor values for the Proteasome-Glo™ Cell-Based Assay were determined using U266 cells with and without 10µM lactacystin treatment. Cells were dispensed (1,500 cells per 5µl) using a Tecan Freedom EVO® 200 liquid handling system and allowed to equilibrate in a humidified 37°C, 5% CO₂ incubator for 1.5 hours before lactacystin or vehicle was added in 5µl volumes. Following a 105-minute incubation at 37°C, 5% CO₂, the assay plate was equilibrated to ambient temperature before adding the Proteasome Glo™ Cell-Based Reagent (10µl/well). Luminescence was determined using a Tecan GENios Pro® luminometer. Ninety-six wells contained untreated cells, and 96 wells in the same plate contained cells treated with 10µM lactacystin. Z’-factor value = 0.77 for this assay. Z’-factor values are statistical indicators of the dynamic range and variability of assay results. A Z’-factor value of 0.5–1.0 is indicative of a high-quality assay (9).
Luminescent Cell-Based Proteasome Assay

Summary

The Proteasome-Glo™ Cell-Based Assay combines the sensitivity of our luminescent protease assays with the simplicity of a single reagent addition. The homogeneous format of this assay eliminates tedious sample processing. The assay enables cell-based measurement of the chymotrypsin-like activity of the proteasome directly in multiwell plates. The simplified method gives results in just 10–15 minutes with a variety of cell types. An “add-mix-measure” protocol was miniaturized to a 384-well plate format and resulted in a Z’-factor value of 0.77. Finally, we were also able to sequentially multiplex this assay with a modified fluorescent Apo-ONE® Homogeneous Caspase-3/7 Reagent to monitor caspase-3/7 induction following epoxomicin inhibitor treatment.

Figure 6. Sequential multiplex to determine proteasome and caspase-3/7 activity. H929 cells (10,000 cells/well) were plated in 90µl per well volumes in a 96-well plate. Following an overnight culture at 37°C, 5% CO₂, cells were incubated with epoxomicin (10µl/well addition) for 1.5 or 4.5 hours at 37°C, 5% CO₂. The plate was removed and allowed to equilibrate to 22°C before 100µl/well of Proteasome-Glo™ Cell-Based Assay Reagent was added. Proteasome-mediated luminescence was measured after 15 minutes as described for Figure 2. Immediately, 20µl/well of a modified concentrated Apo-ONE® Homogeneous Caspase-3/7 Reagent, prepared by diluting the Apo-ONE® Substrate 1:20 into the Apo-ONE® Buffer, was added to the samples. The assay plate was mixed by shaking and incubated at 22°C for an additional 30 minutes before determining caspase-3/7 fluorescence at 485nmEx/527nmEm using a Labsystems Ascent plate fluorometer. GraphPad Prism® was used to obtain EC50 values for epoxomicin.

References


Protocol


Ordering Information

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For Laboratory Use.

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® 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.
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