Molecular Devices

Measurement of Proteasome Inhibition in Live Cells in Molecular Devices Microplate Fluorometers

SPECTRAMAX APPLICATION NOTE #1

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INTRODUCTION

Proteins inside eukaryotic cells exist in a dynamic state, in a highly-regulated balance between synthesis and degradation. Whereas protein synthesis is well-understood after decades of study, major advances in our knowledge of protein degradation have occurred only in the last two decades. As a result, the 2004 Nobel prize in chemistry was awarded to Aron Ciechanover, Avram Hershko and Irwin Rose for their discovery of ubiquitin-mediated proteolysis, an ATP-dependent process where unwanted proteins are multiply-tagged with ubiquitin (a 76-amino acid protein).[1] The tagged proteins are then transported to the proteasome for degradation. The proteasome is a massive (2.5 MDa), barrel-shaped protein complex inside all eukaryotic cells (and some bacteria) that consists of a tunnel-like core with a cap at each end. (See Figure 1.) The caps (regulatory complexes) recognize and bind targeted proteins and inject them into the central core where the proteins are successively degraded into short peptides.

Numerous cellular processes regulated by ubiquitin-mediated proteolysis include cell cycle, differentiation, DNA repair and transcription, stress response, neuronal morphogenesis, cell surface receptor modulation, secretion, regulation, long-term memory, circadian rhythms and immune response.[2] Defects in ubiquitin-mediated proteolysis are implicated in the pathogenesis of many human diseases, including a variety of cancers. Thus it is not surprising that this has become the target for development of drugs against various diseases. One drug, already in clinical trials, is the proteasome inhibitor Velcade® which is approved for treatment of multiple myeloma.[1,3]

BD Biosciences Clontech has recently introduced the BD Living Colors™ HEK 293 ZsGreen Proteasome Sensor Cell Line that allows noninvasive monitoring of proteasome activity.[4] It was obtained by stably transfecting HEK 293 cells with the Proteasome Sensor Vector (pZsProSensor-1). The Protein Sensor Vector encodes a destabilized version of a green fluorescent protein, (ZsGreen; zFP506.1) that is degraded by the proteasome without the requirement for ubiquitin modification.[5] To convert ZsGreen into a proteasomal substrate, its C-terminus was fused to a specific degradation motif that targets the fusion for removal by the proteasome.

Under normal conditions, the fluorescence of the cells is very low because ZsProSensor-1 protein is rapidly degraded. However, under conditions where proteasome activity is inhibited, fluorescence increases as the ZsProSensor-1 protein accumulates. This live cell assay is highly sensitive and can be monitored by fluorescent microscopy,

A Proteasome (Figure 1)

Image courtesy of the U.S. Department of Energy, Genomics: GTL Program, http://www.ornl.gov/hgmis.

flow cytometry, and microplate fluorometry. Below, we show that inhibition of proteasome activity can easily be measured in the following Molecular Devices microplate fluorometers: The Gemini EM is a benchtop scanning microplate fluorometer with top- and bottom-read capability. The FlexStation® is similar, but with integrated fluid transfer capability. The SpectraMax® M5 is a scanning multi-detection benchtop reader, also with top- and bottom-read capability. The Analyst® GT is a multimode reader designed for high-throughput screening environments.

MATERIALS

- HEK 293 ZsGreen Proteasome Sensor Cell Line (BD Biosciences Clontech, Cat. #631535)
- Acetyl-leu-leu-norleu-al (ALLN, CalBiochem Cat. #208719)
- Black-Wall Clear-Bottom 96-Well Microplate (Costar Cat. #3603)
- Gemini EM Scanning Microplate Fluorometer (Molecular Devices)
- SpectraMax M5 Multi-Detection Reader (Molecular Devices)
- FlexStation Scanning Benchtop Fluorometer and Integrated Fluid Transfer Workstation (Molecular Devices)
- Analyst GT Multimode Reader (Molecular Devices)

METHODS

The stock solution of ALLN (a well-characterized proteasome inhibitor) was prepared by dissolving in DMSO to a concentration of 10 mM. The HEK 293 ZsGreen Proteasome Sensor Cells were cultured in DME + 10% FBS + 1% Pen/Strep/L-glutamine + 200 µg/mL of G418. They were seeded overnight in 100 µL at a density of 30,000 cells/well in a Costar black-wall /clear-bottom 96-well plate. The cells were then treated with ALLN at doses of 0, 0.1, 0.3, 1, 3, 10 and 100 µM for 20 hours (N=12/group). The green fluorescence of ZsProsensor-1 in non-treated and treated cells was measured from the bottom in the readers.

Instrument settings

Gemini EM, FlexStation: Ex/Em = 484/510 with a 495 nm emission cutoff filter and PMT set to Auto.

SpectraMax M5: Ex/Em = 484/525 with a 515 nm emission cutoff filter and PMT set to Auto.

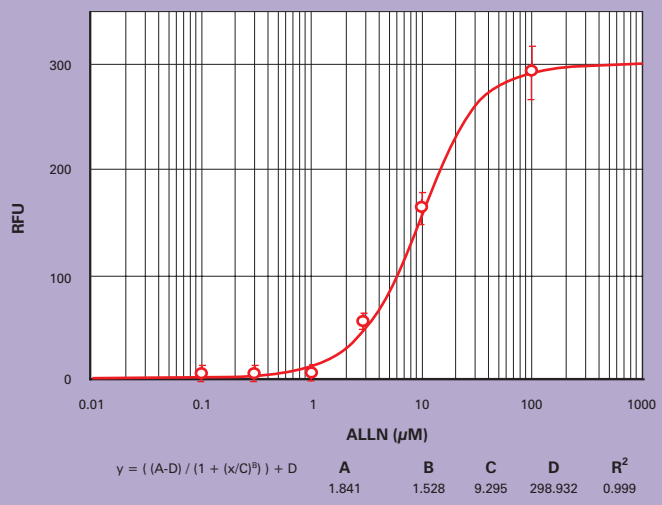
Analyst GT: Ex/EM band-pass filters were 485-20 and 530-25. A 50% transmission dichroic mirror was used. The sensed volume was focused at 1 mm above the well bottom.

RESULTS

ALLN inhibited proteasome activity in a dose-dependent manner, as expected (Figure 2a-2c). (ALLN is a peptide aldehyde that inhibits the proteasome's chymotrypsin activity and prevents the complex from degrading the ZsProsensor protein, thus allowing it to accumulate and causing an increase in the green fluorescence signal.) The dose-response curves from the three instruments were virtually superimposable if normalized. The EC_{50} of ALLN was approximately 10 µM in all instruments. This value is similar to the reported EC_{50} value for ALLN inhibition of an uncharacterized protease g-secretase in HEK-293 cells.[6]

The Z′ factor is a widely-used parameter to indicate the power of a given assay method to distinguish between negative and positive controls. In general, an assay is considered valid if the is Z′ factor at the maximal response concentration (EC_{100}) is > 0.5.[7] In the present experiment, the Z′ factor at the EC_{50} was 0.54-0.66. This Z′ factor at a concentration lower than the EC_{100} indicates a powerful assay.

Proteasome Inhibition on Gemini EM/SpectraMax M5 Readers (Figure 2a)



Proteasome inhibition as a function of ALLN concentration in the HEK 293 ZsGreen Proteasome Sensor Cell Line measured by Molecular Devices Gemini EM or SpectraMax M5 microplate fluorometers. (This plot was obtained on a Gemini EM system, but subsequent experiments with a ZsGreen containing cell line showed the SpectraMax M5 and Gemini EM systems to be comparable in measurement of intracellular ZsGreen.) The Z′ factors ranged from 0.54 to 0.66 for at the 10 µM dose level.

CONCLUSIONS

The proteasome sensor is well-suited for high-throughput screening of candidate modulators of proteasome activity and the assay can easily be run in Molecular Devices microplate fluorometers.

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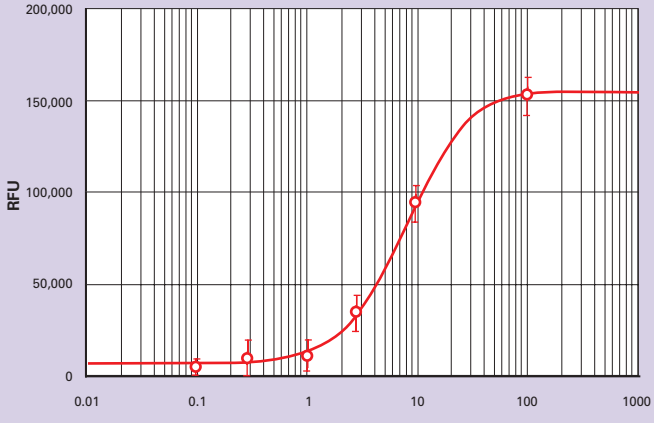
Proteasome Inhibition on FlexStation Reader (figure 2b)

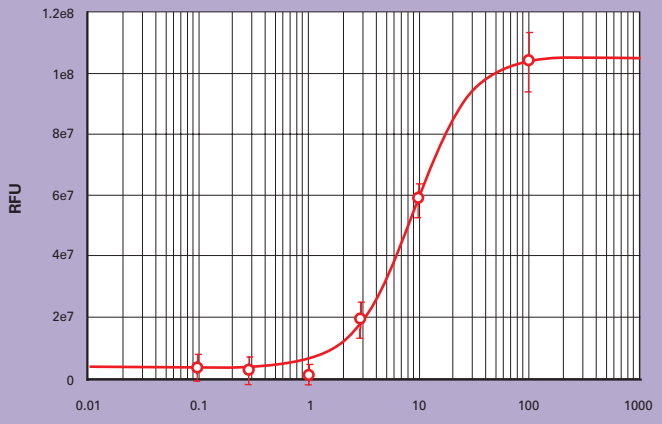


$y = ((A-D) / (1 + (x/C)^B)) + D$

A	B	C	D	R^2
6303.396	1.523	7.982	1.56e5	0.999

Proteasome inhibition as a function of ALLN concentration in the HEK 293 ZsGreen Proteasome Sensor Cell Line measured Molecular Devices FlexStation microplate fluorometers. The Z´ factors ranged from 0.54 to 0.66 for at the 10 μM dose level.

Proteasome Inhibition in Analyst GT System (Figure 2c)



$y = ((A-D) / (1 + (x/C)^B)) + D$

A	B	C	D	R^2
2.68e6	1.673	8.932	1.05e8	0.995

Proteasome inhibition as a function of ALLN concentration in the HEK 293 ZsGreen Proteasome Sensor Cell Line measured Molecular Devices Analyst GT. The Z´ factors ranged from 0.54 to 0.66 for at the 10 μM dose level.

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