PR-619, A Novel small molecule reversible inhibitor of deubiquitylases (DUBs).

Suresh Kumar, Senior Scientist
Progenra, Inc.

BACKGROUND

Ubiquitin and Polyubiquitylation in Cellular Processes

The post translational modification of proteins with ubiquitin is an important signal in the regulation of a variety of cellular processes. Ubiquitin is a small protein that is conjugated via its C-terminus to the ε-amino groups of lysine residues on target proteins in either monomeric form or as polymeric chains. Lysine48-linked polyubiquitylation leads to proteasome-mediated degradation of the target protein. Polyubiquitin modification is a reversible process; these chains are removed by a class of proteases known as deubiquitylases (DUBs). The complex nature of this enzymatic pathway presents a major challenge in understanding the physiological role of ubiquitin and associated enzymes in the cell.

PR-619 as a novel small molecule reversible inhibitor of DUBs

Cell permeable, small molecule inhibitors of ubiquitin pathway enzymes such as DUBs will have high utility for studying the cellular functions of these enzymes. LifeSensors has recently introduced PR-619 (licensed from Progenra, Inc.), a small molecule that reversibly inhibits DUBs in *in vitro* assays. PR-619 can be used in several application, including *in vivo* treatment of cell lines and tissue slices or added to the lysis buffer to generate cell extracts for further analyses. PR-619 is also recommended for pulldown assays using TUBE (Tandem Ubiquitin Binding Entity) products from LifeSensors (Cat. Nos UM101, UM102, UM201, UM202, UM203, UM401 and UM402). We also recommend to use PR-619 for the protection of tagged polyubiquitylated proteins from endogenous DUBs (non-specific binding to the affinity resin) during purification from cell and tissue lysates . Here, we provide a protocol for the detection of accumulating polyubiquitin conjugates in cells in the presence of PR-619. We recommend that the optimum inhibitor concentration, time of treatment and plating density of cells be determined for individual cell types being tested. Treatments for longer than 24hrs at higher concentrations may result in toxicity.

EFFECT OF PR-619 ON GLOBAL CELLULR POLYUBIQUITYLATION

A 10mM stock solution of PR-619 was made by dissolving 5mg of PR-619 in 2.24ml of DMSO, aliquots were prepared and stored at -80°C. HCT-116 cells and HEK 293 cells were grown in 6-well plates to 80-90% confluency and treated with of PR-619 over the range 0 to 50µM for various periods of time (0 to 24 hrs). DMSO alone at 0.5% (v/v) was used as the vehicle control. After treatment, cells were harvested by scraping in ice cold PBS and collected by centrifugation (1,500xg). Cell pellets were subjected to one freeze-thaw cycle (-80°C) and suspended in cold cell lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1% NP-40, 10% Glycerol, 1mM PMSF, protease inhibitor cocktail (Sigma, Cat. No. P-8849) and 20µg/ml aprotinin). Total cell lysates (25µg) were boiled in the presence of 1X Laemmli sample buffer and separated on 4-20% gradient polyacrylamide SDS gels, transferred to a PVDF membrane and probed with anti-ubiquitin antibody (Sigma, U5379). To confirm equivalent loading, the blot was stripped and re-probed with anti-β-actin antibody (Sigma, A5441).

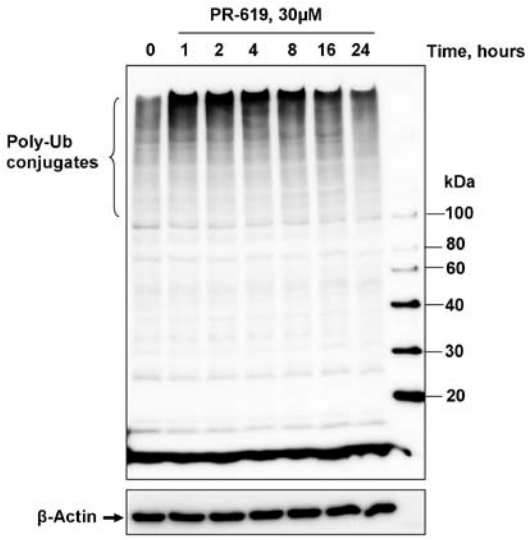

Figure 1. HCT-116 cells were treated with 30µM PR-619 for indicated times. Samples (25µg of cell lysates) were processed for Western blot analysis and immunoblotted with anti-ubiquitin antibody (upper panel). The blot was stripped and re-probed with anti-β-actin antibody (lower panel).

The accumulation of polyubiquitylated protein aggregates (poly-Ubi) is substantially increased starting at 1 h after the treatment with PR-619. The slight decrease in immunoreactive polyubiquitylated proteins could be due to a toxic effect of PR-619 on cultured cells after 24 hr at 30 µM. Alternatively, it may be a result of the reversible inhibition by PR-619.

ADDITIONAL REAGENTS REQUIRED

1. Cell lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1% NP-40, 10% Glycerol, 1mM PMSF)
2. Protease cocktail inhibitor (Sigma, Cat. No. P-8849)
3. Aprotinin (Sigma, Cat.No.A1153)
4. Bio-Rad Protein Assay Kit (Cat. No. 500-006)
5. Rabbit polyclonal anti-ubiquitin antibody (Sigma, Cat. No. U5379)
6. Mouse anti-β-actin antibody (Sigma, Cat. No.A5441)
7. Phosphate Buffered Saline, pH 7.5 (PBS)

REFERENCES

1. Pickart, C.M. and Eddins, M.J. (**2004**). "Ubiquitin: structures, functions, mechanisms." *Biocim biophys Acta*. 1695 (1-3): 55-72.

2. Wilkinson, K. D. (**2000**). "Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome." *Semin Cell Dev Biol* 11(3): 141-8.

3. Ciechanover, A. (**2003**) "The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting." *Biochem Soc Trans* 31(2): 474-81.

4. Xufan Tian, Nigora Isamiddinova, RJ Peroutka, Seth Goldenberg, Michael Mattern, Benjamin Nicholson, Craig Leach (**2010**) Characterization of selective isopeptidase inhibitors using a fluorescence-based multiplex assay format (Submitted)

About LifeSensors, Inc.

LifeSensors is a biotechnology company located 35 miles west of Philadelphia, Pennsylvania, USA. Founded in 1996, LifeSensors has developed a number of innovative protein expression technologies that enable efficient translation of the genome into proteome.

LifeSensors is well-known for its innovations in an important family of proteins consisting of ubiquitin and ubiquitin-like proteins (UBL) such as SUMO (Small Ubiquitin-like MOdifier).

LifeSensors has been granted several patents to cover the use of SUMO and other UBLs as gene fusion tags to improve the expression and purification of recombinant proteins. Additional patent applications are in various stages of review. Currently, LifeSensors is expanding its protein production capabilities and is developing protein micro array for drug discovery and diagnostics.

PR-619 was licensed by LifeSensors, Inc. from Progenra, Inc. For information on obtaining a license for commercial purposes, contact: Marc Scholtyssek, Sales Manager, LifeSensors, Inc., Malvern PA 19355, Phone: 610.644.8845 ext 305.

LifeSensors, Inc., 271 Great Valley Parkway, Malvern PA 19355
(p) 610.644.8845 (f) 610.644.8616
techsupport@lifesensors.com • www.lifesensors.com • sales@lifesensors.com