PR-619

THE INHIBITION OF UBIQUITIN AND UBIQUITIN-LIKE ISOPEPTIDASES THROUGH THE USE OF A CELL PERMEABLE SMALL MOLECULE

White Paper
August 2010

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Ubiquitin and Polyubiquitylation in cellular processes

The post-translational modification of proteins with ubiquitin is an important signal in the regulation of the variety of cellular processes. Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to ε-amine groups of lysine residues on target proteins in either monomer form or as polymer chains [1]. Lysine 48-linked (K48) polyubiquitylation is typically associated with proteasome-mediated degradation of the target protein. Polyubiquitin modification is a reversible process; these chains are removed by a class of protease known as deubiquitylases (DUBs) [2, 3]. The human genome encodes nearly 100 putative DUBs that are classified into five different families. Four of these families consist of cysteine proteases with reactive thiols at their active sites: the ubiquitin C-terminal hydrolases (UCHs) family, the ubiquitin specific proteases (USPs) family, the ovarian tumor (OUT) domain family, and the Machado-Joseph domain (MJD) family. The fifth family of DUBs is distinct in consisting of a number of zinc-dependent metalloproteases, and as such is referred to as the JAB1/MPN/Mov34 metalloenzyme (JAMM) domain containing family [4,5].

In addition to ubiquitin, there are additional small protein adducts that impact cellular fate/function of target proteins post-translationally. These ubiquitin-like proteins (Ubls) include Small Ubiquitin like Modifiers (SUMO), neuronal precursor cell-expressed developmentally down-regulated gene 8 (NEDD8), and interferon stimulated gene 15 (ISG15), and like ubiquitin are reversibly conjugated to target proteins. Ubl conjugation is critical in directing intracellular compartmentalization of proteins, signal transduction and modulating cellular immune responses. Similar to DUBs, Ubl-isopeptidases have been identified that catalyze the removal of Ubls from target proteins. Some of the more well characterized Ubl-isopeptidases are SENtrin specific proteases (SENPs), deneddylase1 (DEN1), as well as USP18 and its murine homologue UBP43 that remove SUMO, Nedd8 and ISG15 conjugations respectively [6-11].

Several Ub/Ubl isopeptidases are implicated in various diseases including cancer, neurological disorders and infectious diseases [12, 13]. DUBs such as USP7, USP20, USP33, and USP2a are considered attractive cancer therapeutic targets [12]. The presence of a defined catalytic active site makes the targeting of these enzymes conceivable and many pharmaceutical companies, as well as academic laboratories, are developing small molecule inhibitors for therapeutic use and as tool compounds for research purposes.

PR-619 as a novel small molecule inhibitor of Ub/Ubl isopeptidases

Cell permeable, inhibitors of ubiquitin pathway enzymes such as Ub/Ubl isopeptidases have tremendous potential for use in a variety of applications, such as studying the cellular functions of these enzymes in their native environs and as tool compounds in drug discovery for these targets. In order to fill this unmet need, LifeSensors has recently licensed the small molecule PR-619 from Progenra, Inc[14. The utility of this molecule is defined by its broad specificity for a number of Ub/Ubl isopeptidases, the reversible mechanism of inhibition, as well as its cell permeable nature.

This paper describes the characterization of PR-619 in the CHOP Reporter System, providing a template for the use of this small molecule as a tool compound for high throughput screening (HTS)

and discovery of reversible inhibitors of Ub/Ubl isopeptidases. We also demonstrated that PR-619 protects the degradation of polyubiquitinated proteins in cell lysates in the absence of the thiol reactive compound N-Ethylmaleimide (NEM), improving detection limits for studies of the global ubiquitylation state of the cell. Finally, we describe the inhibition of isopeptidase activity in whole tissues with this compound, demonstrating the cell permeable nature of PR-619.

The application of PR-619 to the inhibition of Ub/Ubl isopeptidases

PR-619 Inhibits Isopeptidases *in vitro* with Broad Specificity

Inhibition of Ubl isopeptidases *in vitro* by PR-619 was measured using LifeSensors' CHOP Reporter System (Cat# PR1001) as described previously [15]. While PR-619 selectively inhibited Ubl-isopeptidases (**Figure 1, Table 1**) while it does not inhibit the chymotrypsin like activity (CT-L) of the 20S proteasome. In addition, as the both CHOP1 and CHOP2 Reporter Systems are coupled assays that are dependent upon the conversion of a reporting enzyme from an inactive state, PR-619 was tested against the active forms of these enzymes, with no detectable inhibition towards either phospholipase A2 (CHOP1) or enterokinase light chain (CHOP2). The determined IC50s for all isopeptidases were comparable, illustrating the broad applicability of this tool compound without interfering with proteasomal activity. These data demonstrate that, PR-619 is a useful compound for the establishment and validation of *in vitro* Ub/Ubl isopeptidase assays, as well as for the discovery of novel inhibitors for these targets.

Preservation of polyubiquitylated proteins during cell lysis

The addition of protease inhibitor cocktail to the lysis buffer during cell lysis has become a standard practice to ensure integrity of proteins for downstream analysis and characterization. However, researchers in the ubiquitin field face additional challenges to preserve the integrity of the polyubiquitylated protein of interest either within the live cells or during cell lysis due to a very dynamic nature of ubiquitylation. Following cell lysis, ubiquitylated proteins are susceptible to degradation through activity by both DUBs and the proteasome. Many protease inhibitor cocktails do not specifically target DUBs, and classical reagents used to target DUBs (e.g. NEM) irreversibly inhibit or otherwise modify a broad range of enzymes within the cell. Thus, novel DUB inhibitors such as PR-619 with abroad DUB specificity, provide an invaluable tool for studying the ubiquitin-proteasome pathway.

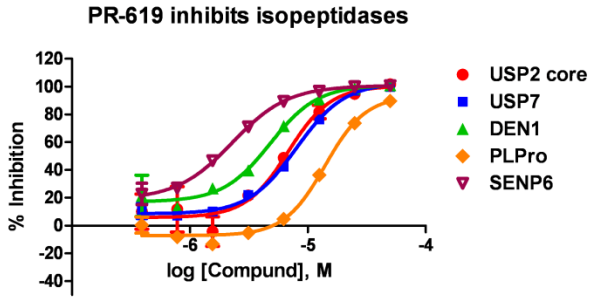

Figure 1. Inhibition curves of PR-619 against various ubiquitin and ubiquitin-like isopeptidases

To demonstrate the ability of PR-619 to abrogate the deconjugation of polyubiquitylated proteins, HEK293 were subjected to one freeze-thaw cycle and cell lysates were prepared at 4° in the lysis buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% NP40, 10% glycerol, 1mM PMSF in the presence of Protease Inhibitor Cocktail [Sigma-Aldrich]) containing either 50µM PR-619 or 0.5% DMSO as a vehicle control. Cell lysates were pre-cleared by centrifugation and increasing amounts of total protein were subjected to pull-down with pre-equilibrated Agarose-TUBE1 beads (LifeSensors Cat. #U401). TUBEs (Tandem Ubiquitin **B**inding **E**ntities) have been shown to selectively bind polyubiquitylated proteins with high affinity [16]. After incubation of cell lysates with Agarose-TUBE1 (4°C, 1hr), beads were collected by low speed centrifugation and washed with TBS, pH 7.5 containing 10% glycerol to minimize non-specific binding. Polyubiquitylated proteins were eluted from the resin by SDS-PAGE sample buffer at 90°C and analyzed by SDS-PAGE followed by Western blot analysis along with the "input" (25µg),

Enzyme	IC_{50} ± S.D. (µM)
USP2 core	7.2±1.6
USP7	7.6±3.1
DEN1	5.0±1.2
PLPro	1.4±0.4
SENP6 core	2.4±0.2
20S Proteasome (CT-L)	>50
Phospholipase A_2 (PLA_2)	>50
Enterokinase light chain (EKL)	>50

Table 1. PR619 selectively inhibits Ub/Ubl isopeptidases

"unbound fraction" (1/10th of 0.25mg lysates used in the pull-down) and "mock"-Agarose pull-down (1mg of lysate). Finally, polyubiquitylated proteins were identified using Biotin-TUBE1 (LifeSensors, Cat# UM301), followed by incubation with Strepatvidin-HRP (Rockland) for the detection.

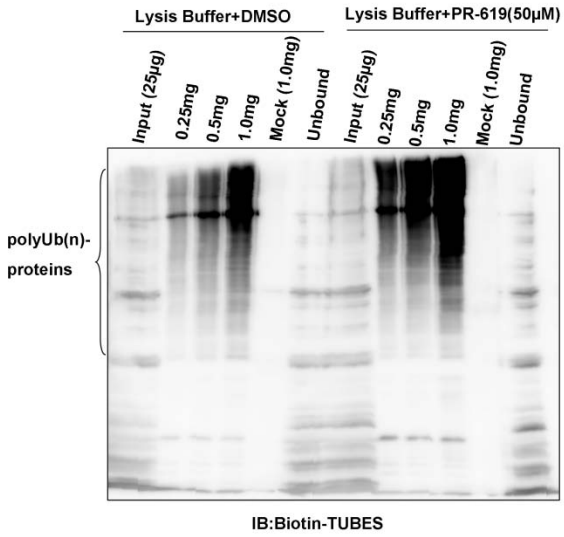

Figure 2. PR619 protects polyubiquitin chains in cell lysates

As shown in **Figure 2**, the addition of 50μM PR-619 to the lysis buffer significantly enhanced the amount of polyubiquitin conjugates isolated and detected from HEK293 lysates compared to the vehicle control. Lysates (starting from 0.25mg of total protein) produced a strong signal in the high molecular weight range, indicating the presence of polyubiquitin conjugates. Increased amounts of polyubiquitylated proteins after the treatment with PR-619 was proportional to the total amount of protein tested. These results clearly indicate that PR-619 treatment significantly increases the yield of recovered polyubiquitin conjugates from cell lysates and maintains polyubiquitin chain integrity, which is critical for downstream protein analysis such as Western blotting or proteomic analysis by liquid chromatography-mass spectroscopy (LC-MS).

PR619 increases overall cellular polyubiquitylation

In addition to the ability to inhibit DUB activity *in vitro* in cell lysates, PR-619 can be used to inhibit intracellular DUBs in living cells due to its cell permeable nature for the *in vivo* experiments. HEK293 cells were grown in 60mm dishes to 80-90% confluency, and treated with either 50μM PR-619 or 0.5% DMSO vehicle control for 2hrs. Cell lysates were generated as above and total cell lysates (25μg) were subjected to SDS-PAGE prior to detection of polyubiquitylated proteins with Biotin-TUBES (LifeSensors, Cat. # UM301).

To control for equivalent loading, the blot was stripped and re-probed with anti-β-actin antibody (Sigma, A5441).

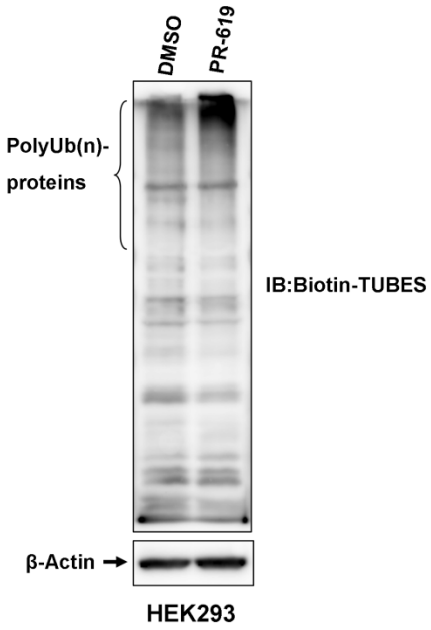

Figure 3. PR619 (50μM, 2hrs) increases accumulation of polyubiquitylated proteins in HEK293 cells

As shown in **Figure 3**, treatment of HEK293 cells for 2hrs with 50μM PR-619 results in robust accumulation of polyubiquitylating conjugates relative to vehicle control. Given the high molecular weights of the conjugates, it is likely that they are mostly comprised of polyubiquitylated proteins. Thus, PR-619 can be used *in vivo* for cell treatment and *in vitro* to treat tissue and cell lysates, to enable efficient enrichment of polyubiquitylated proteins for subsequent analyses such as LC-MS and Western blot. Even though PR-619 does not directly inhibit the chymotrypsin-like catalytic activity of the proteasome, it might inhibit proteasome functions indirectly, since proteasomes require ubiquitin removal (by associated deubiquitylase) from target proteins prior to translocating them to the catalytic core for degradation [17]. Therefore, using PR-619 might be beneficial for studies investigating the interaction of polyubiquitylated proteins with proteasomes.

We recommend that the optimum inhibitor concentration, time of treatment and plating density of cells be determined for each individual cell type by the end user. Treatment with PR619, an isopeptidase inhibitor with a broad specificity for longer times at higher concentrations (>50 μM) may result in toxicity.

Inhibition of Ub/Ubl Isopeptidases in Whole Tissue

In contrast to cells in culture, intact tissue samples (blocks) used for the physiological experiments often present significant barrier to the penetration of small molecule inhibitors and drugs [18, 19]. The ability of a compound to penetrate these tissue samples would provide a great opportunity to utilize this tool in assays such as biomarker analyses. Inhibition of ubiquitin isopeptidase activity in tissues would be expected to enhance polyubiquitylation of target proteins, similar to that observed in cells grown in culture. The ability of PR-619 to penetrate the intact rodent brain tissue was evaluated by measuring the residual isopeptidase activity in tissue homogenates after the extensive wash of the inhibitor. Furthermore, in separate experiments, reversibility studies were carried out using tissue homogenates treated with PR-619.

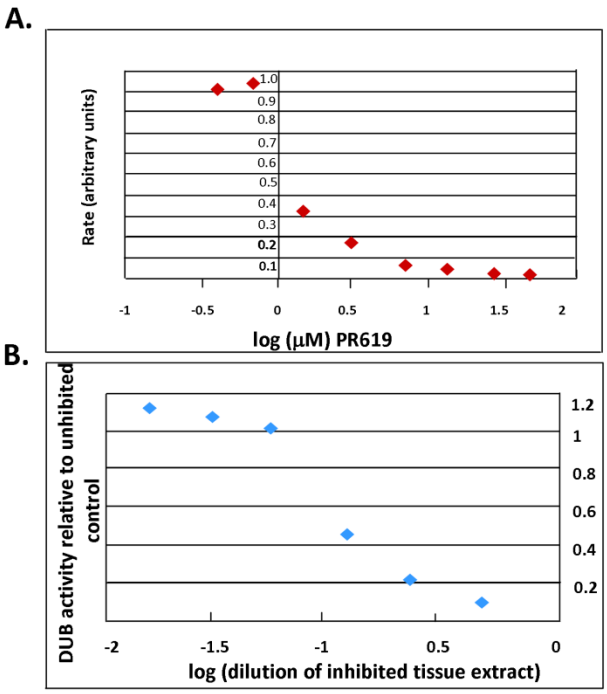

Figure 4. PR619 inhibits DUB activity in intact rodent brain samples (A) and tissue homogenates (B)

To test the ability of PR-619 to penetrate intact tissue, rodent brain samples were treated (1hr) with increasing concentrations of PR619 (up to 50 µM) followed by washes in PBS for 1 hr to remove residual inhibitor. Tissue samples were homogenized in the Assay buffer (20mM Tris buffer, pH 8.0, 150mM NaCl, 2mM CaCl, 2mM β-mecaptoethanol, 0.05% CHAPS) by sonication in a cup horn sonicator. Tissue extracts were clarified by centrifugation at 12,000K for 5 min at 4°C, and DUB activity was measured using the CHOP2 Reporter System (LifeSensors, Cat. # PR1101) and are presented relatively to untreated control samples processed in parallel. The relative DUB activity in tissue lysates indicates a clear dose response for PR-619, confirming the ability of this compound to penetrate and irreversibly inhibit isopeptidases *in situ* (**Figure 4A**). Additional experiments are needed to identify a precise mechanism of DUB inhibition in this experimental model (i.e. pharmacokinetic of the inhibitor).

To investigate the reversibility of PR-619 in tissue extract, *untreated* rodent brain tissue was used to prepare homogenates in the Assay buffer. Tissue extracts were clarified by centrifugation at 12,000K for 5 min at 4°C and incubated with 50 µM PR-619. Serially diluted samples containing PR-619 were then assayed for DUB activity as described above. As demonstrated in **Figure 4B,** tissue samples treated with PR-619 exhibit full recovery of DUB activity, relative to control samples, upon dilution suggesting that PR-619 inhibition is fully reversible.

These studies demonstrate that PR619 is capable of penetrating into rodent brain tissue in situ and can be used for the inhibition of DUBs using whole tissue preparations. In addition, PR619 reversibly inhibits DUB activity *in vitro* in cell and tissue extracts making this compound a useful tool for studying a dynamic process of ubiquitylation.

Frequently Asked Questions

Q: Is PR-619 selective?

A: PR-619 inhibits all Ub/Ubl isopeptidases tested to date. It has negligible inhibitory activity towards serine proteases, metalloproteases, aspartic acid, threonine and most other families of cysteine proteases.

Q: Is PR-619 cell permeable?

A: Yes, PR-619 is a cell permeable small molecule.

Q: What is the recommended concentration for usage in cell lysis buffer?

A: It is recommended that PR-619 be added at a concentration of 50µM to the lysis buffer of choice prior to cell lysis and harvest to ensure maximal inhibition of isopeptidase activity.

Q: Is PR-619 cytotoxic?

A: Yes PR-619 is cytotoxic against a number of human cancer cell lines tested. It is strongly suggested that the end user empirically determine the dose of PR-619 required for DUB inhibition with minimal toxicity for individual cell lines.

Q: What is the recommended concentration for treating cells?

A: PR-619 exhibits time and concentration dependent cytotoxic effects on human cancer and normal cell lines tested. In a 72 hour cytotoxicity assay, PR-619 showed cytotoxicity against HCT116 (6.5µM EC50) and WI-38 cells (5.3µM EC50). The optimal concentration of compounds for treating cells will vary depending on the cell lines, cell density and the time of treatment. We recommend that a range of concentrations be tested for individual cell lines at different plating densities.

Q: Is PR-619 stable?

A: PR-619 is stable in DMSO when stored at -80^{o}C.

References

1. Ciechanover, A., The ubiquitin proteolytic system and pathogenesis of human diseases: a novel platform for mechanism-based drug targeting. Biochem Soc Trans, 2003. **31**(2): p. 474-81.
2. Wilkinson, K.D., DUBs at a glance. J Cell Sci, 2009. **122**(Pt 14): p. 2325-9.
3. Wilkinson, K.D., Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. Faseb J, 1997. **11**(14): p. 1245-56.
4. Reyes-Turcu, F.E., K.H. Ventii, and K.D. Wilkinson, Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu Rev Biochem, 2009. **78**: p. 363-97.
5. Reyes-Turcu, F.E. and K.D. Wilkinson, Polyubiquitin binding and disassembly by deubiquitinating enzymes. Chem Rev, 2009. **109**(4): p. 1495-508.
6. Bornstein, G., D. Ganoth, and A. Hershko, Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate. Proc Natl Acad Sci U S A, 2006. **103**(31): p. 11515-20.
7. Drag, M. and G.S. Salvesen, DeSUMOylating enzymes--SENPs. IUBMB Life, 2008. **60**(11): p. 734-42.
8. Malakhov, M.P., O.A. Malakhova, K.I. Kim, K.J. Ritchie, and D.E. Zhang, UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J Biol Chem, 2002. **277**(12): p. 9976-81.
9. Malakhova, O.A., K.I. Kim, J.K. Luo, W. Zou, K.G. Kumar, S.Y. Fuchs, K. Shuai, and D.E. Zhang, UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. Embo J, 2006. **25**(11): p. 2358-67.
10. Mikolajczyk, J., M. Drag, M. Bekes, J.T. Cao, Z. Ronai, and G.S. Salvesen, Small ubiquitin-related modifier (SUMO)-specific proteases: profiling the specificities and activities of human SENPs. J Biol Chem, 2007. **282**(36): p. 26217-24.
11. Wu, J.T., H.C. Lin, Y.C. Hu, and C.T. Chien, Neddylation and deneddylation regulate Cul1 and Cul3 protein accumulation. Nat Cell Biol, 2005. **7**(10): p. 1014-20.
12. Nicholson, B., J.G. Marblestone, T.R. Butt, and M.R. Mattern, Deubiquitinating enzymes as novel anticancer targets. Future Oncol, 2007. **3**(2): p. 191-9.
13. Colland, F., The therapeutic potential of deubiquitinating enzyme inhibitors. Biochem Soc Trans. **38**(Pt 1): p. 137-43.
14. Xufan Tian, N.I., RJ Peroutka, Seth Goldenberg, Michael Mattern, Benjamin Nicholson, Craig Leach, Characterization of selective isopeptidase inhibitors using a fluorescence-based multiplex assay format. (Submitted), 2010.
15. Nicholson, B., C.A. Leach, S.J. Goldenberg, D.M. Francis, M.P. Kodrasov, X. Tian, J. Shanks, D.E. Sterner, A. Bernal, M.R. Mattern, K.D. Wilkinson, and T.R. Butt, Characterization of ubiquitin and ubiquitin-like-protein isopeptidase activities. Protein Sci, 2008. **17**(6): p. 1035-43.
16. Hjerpe, R., F. Aillet, F. Lopitz-Otsoa, V. Lang, P. England, and M.S. Rodriguez, Efficient protection and isolation of ubiquitylated proteins using tandem ubiquitin-binding entities. EMBO Rep, 2009. **10**(11): p. 1250-8.
17. Zhu, Q., G. Wani, Q.E. Wang, M. El-mahdy, R.M. Snapka, and A.A. Wani, Deubiquitination by proteasome is coordinated with substrate translocation for proteolysis in vivo. Exp Cell Res, 2005. **307**(2): p. 436-51.
18. Tunggal, J.K., D.S. Cowan, H. Shaikh, and I.F. Tannock, Penetration of anticancer drugs through solid tissue: a factor that limits the effectiveness of chemotherapy for solid tumors. Clin Cancer Res, 1999. **5**(6): p. 1583-6.
19. Tannock, I.F., C.M. Lee, J.K. Tunggal, D.S. Cowan, and M.J. Egorin, Limited penetration of anticancer drugs through tumor tissue: a potential cause of resistance of solid tumors to chemotherapy. Clin Cancer Res, 2002. **8**(3): p. 878-84.