Deubiquitylation and protein aggregation: implications for neurodegenerative diseases.

Effect of PR-619, DUB inhibitor, on protein aggregate formation in neural cells

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INTRODUCTION

Filamentous protein inclusions are observed in a number of neurodegenerative diseases. They develop before the onset of cell degeneration and are characterized by the presence of ubiquitin, a variety of heat shock proteins (HSP) and often a specific cellular protein.

Pathological inclusions of the microtubule associated protein (MAP) tau are prominent in Alzheimer's disease, progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). These inclusions are prominent in nerve cells and glia, and specifically originate in oligodendrocytes. Heat shock proteins prevent protein aggregation and support proteolytic degradation by targeting non-reparable proteins to the ubiquitin-proteasome system. If this system is overwhelmed or defective, protein aggregates may be formed. For proteasomal degradation, polyubiquitylated proteins enter the 20S particle of the proteasome and have to be de-ubiquitylated and unfolded before degradation. Deubiquitinating enzymes (DUBs) are a class of isopeptidases that cleave ubiquitin from target proteins, including other ubiquitins. Inhibition of DUBs may lead to the dysregulation of cellular homeostasis and have pathological consequences.

To asses the effects of DUB inhibition on protein aggregate formation and cell survival, we have used an oligodendroglial cell culture model systems, namely OLN-t40 cells, stably transfected to express the longest human isoform of MAP tau. To assess the effects of DUB inhibition, cells were incubated with PR-619 (LifeSensors Inc., USA), a cell-permeable, non-selective, reversible inhibitor of ubiquitin and ubiquitin-like isopeptidases.

About PR-619

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. PR-619 has a broad specificity for a number of Ub/Ubl isopeptidases, the reversible mechanism of inhibition, as well as its cell permeable nature. 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

http://www.lifesensors.com/pdf/PR619_white_paper-1.pdf
http://www.lifesensors.com/product-family-desc.php?id=59

MATERIALS & METHODS

Cell culture

Cells were cultured at 37°C, 10% CO_2 in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin. Medium was changed to 0.5% FCS-DMEM before treatment.

Immunofluorescence analysis

Cells were cultured on PLL coated glass coverslips. After washing with PBS, cells were fixed with methanol, or paraformaldehyde (4%) and permeabilized with Triton X-100 (0.1%). Indirect immunofluorescence was carried out using primary antibodies as indicated. Nuclei were stained by 4´,6-diamidino-2-phenylindole (DAPI) (1.5 µg/ml) included in the mounting medium (Vectashield; Vector Laboratories, CA, USA).

MTT assay

To assess the cytotoxic potential of the compounds, the MTT (Tetrazolium) assay was carried out as described earlier (Richter-Landsberg and Vollgraf, 1998). Cells were plated on PLL-coated 96-microwell cell culture plates (2 x10^4 cells/well) and grown for 7 days. Ten microliters of MTT solution (5 mg/ml in PBS) was added to the wells containing 100 µl medium, and the plates were incubated for 4 h. 100 µl of a solubilization buffer (10% sodium dodecyl sulfate in 0.01 mol/HCl) was added and incubated overnight. Quantification was then carried out with an ELISA reader at 595 nm, using a 655-nm filter as a reference. Data are expressed as percentage of the untreated controls, and values represent the means ±SD of eight microwells each of three independent experiments.

Immunoblot analysis

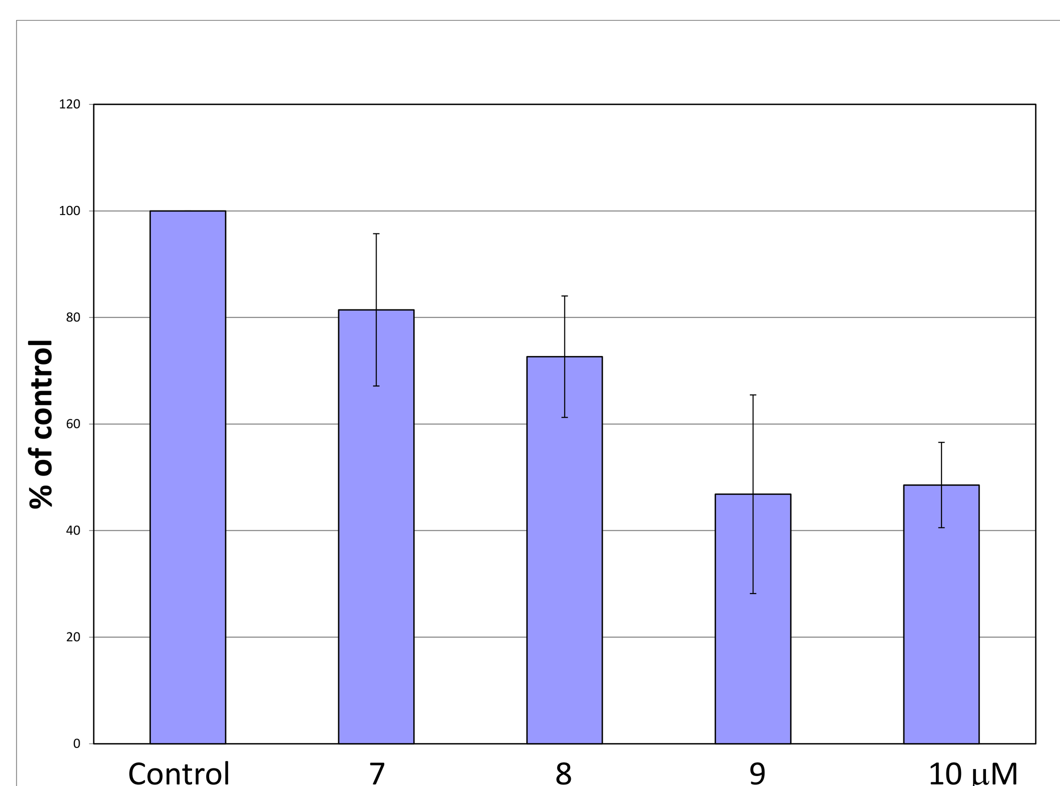
Cells were washed with PBS, scraped off in the sample buffer containing 1% SDS, boiled for 10 min and protein concentration in the samples was determined. For immunoblotting, total cellular extracts were separated by one-dimensional SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked with 5% non-fat milk/TBS-T (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% v/v Tween 20), and incubated with the individual antibodies overnight at 4°C. VU-1 mouse monoclonal antibody (1:2000, LifeSensors, Inc.) was used to detect ubiquitylated proteins. After washing, incubation with HRP-conjugated anti-mouse (1:3000) or anti-rabbit IgG (1:3000) was carried out for 1 h, and immunopositive bands were visualized using the enhanced chemiluminescence kit (ECL, Amersham, Braunschweig).

Sequential extraction

Isolation of soluble and insoluble proteins from cultured cells was performed as described by Dou et al. (2003). Briefly, cells were lysed in a lysis buffer (0.5% Nonidet P-40, 1 mM EDTA, 50 mM Tris HCl, pH 8.0, 120 mM NaCl) supplemented with protease inhibitors mixture (Complete; Roche Diagnostics, Germany) and centrifuged at 15.000 × g to generate supernatant (SN) and pellet (P) fractions.

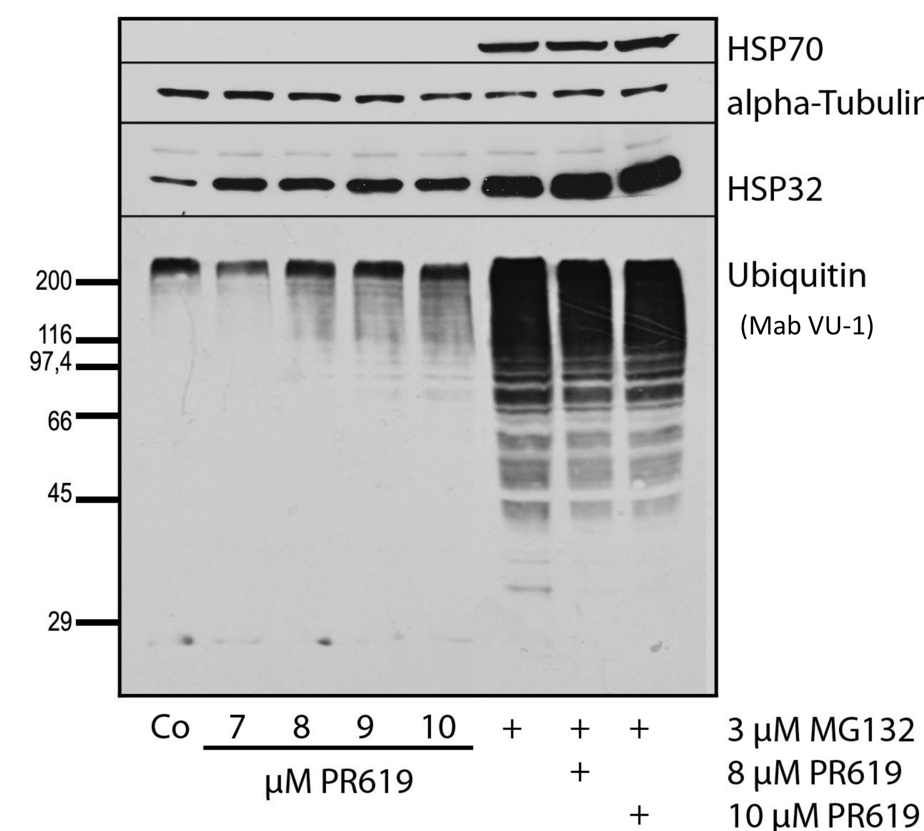
SDS sample buffer was added to each fraction and boiled for 10 min. Equal amounts of total protein from control and treated samples were loaded.

Fig. 1. MTT Cytotoxicity Assay



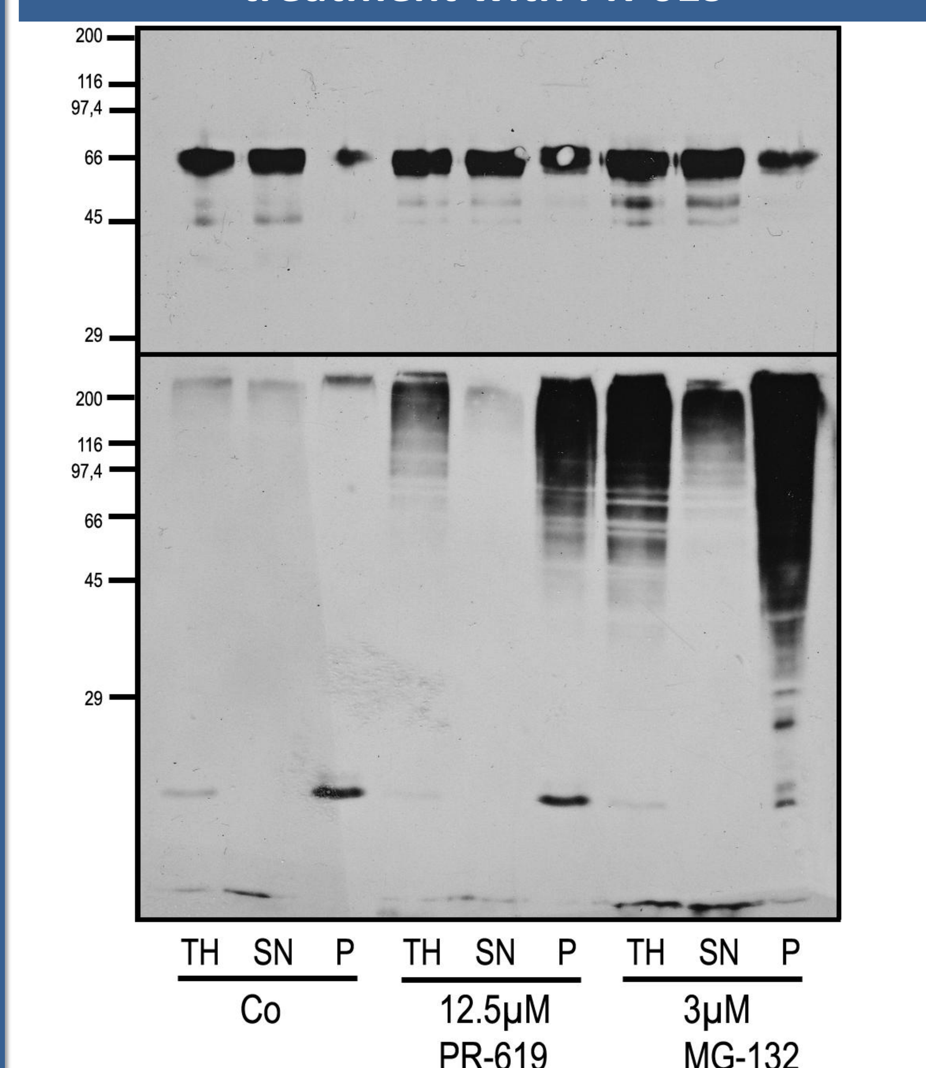
PR-619 induced time- and concentration-dependent cytotoxicity. Half maximal cytotoxicity was evident after 24 h treatment in the range of 9-12 µM.

Fig. 2. PR-619 promotes the accumilation of ubiquitylated proteins in OLN-t40 cells



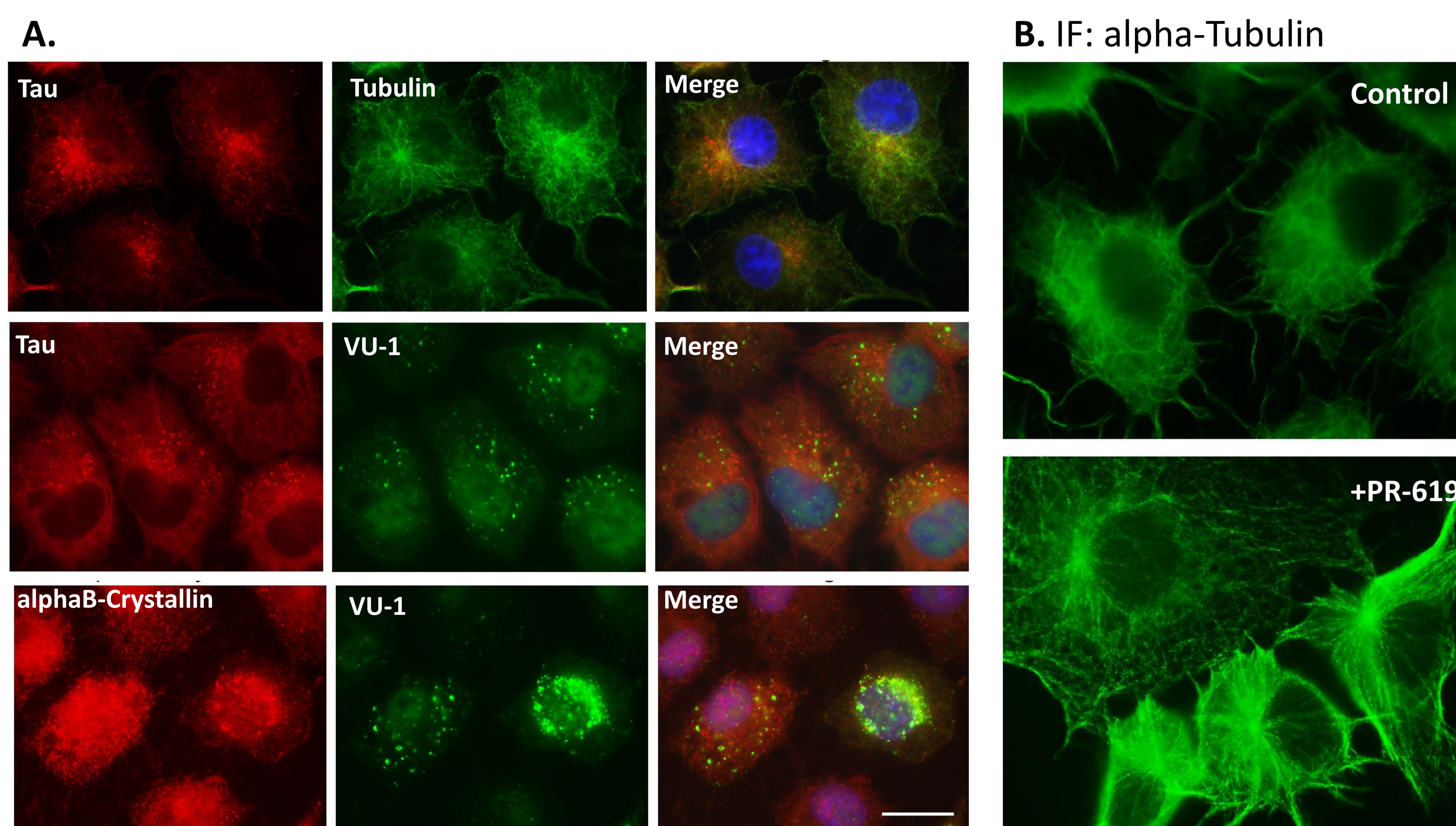
The accumulation of ubiquitinated proteins and the induction of HSP32, an indicator of oxidative stress, was noticable after the treatment with PR-619. As opposed to the treatment with the proteasomal inhibitor MG-132, HSP70 was not upregulated. Cells were treated for 24h either with PR-619, MG-132 or both, as indicated.

Fig. 3. Tau and ubiquitylated proteins are enriched in the insoluble fraction after treatment with PR-619



Cells were treated with 10µM PR-619 for 24 h. **TH,** Total homogenate; **SN,** soluble fraction; **P,** pellet (detergent insoluble fraction).

Fig. 4. Tau and alphaB-crystallin are recruited to ubiquitin-positive inclusions (A) and the microtubule network is impaired (B) after treatment with PR-619



VU-1 ubiquitin MAb (LifeSensors) was used to identify ubiquitylated proteins.
PR-619 treatment: 10 µM for24 h

CONCLUSIONS

- **Inhibition of DUB activity by PR-619 treatment impairs cell morphology , promotes the accumulation of ubiquitylated proteins and leads to protein aggregate formation in OLN-t40 cells.**
- **DUB inhibitors provide a useful tool to further characterize the poorly understood mechanisms of action of DUBs and their dysregulation in neurodegenerative diseases.**