A Novel Cell-Based Drug Discovery Assay for Screening Modulators of Protein Aggregation and Toxicity

Wayne F. Patton[1], Dee Shen[1], Tom Nicholson[2], Jack Coleman[1], Eric Chan[1], Lijun Dai[1], Praveen Pande[1], and Paul Sheppard[2]

[1]Enzo Life Sciences, Farmingdale, NY 11735 and [2]Enzo Life Sciences Ltd, Exeter, UK

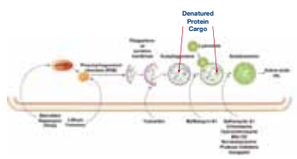
ABSTRACT

Neurodegenerative disorders, such as Huntington's, Parkinson's and Alzheimer's diseases, are characterized by the accumulation of aggregated protein deposits within affected regions of the brain, possibly signifying a malfunction in the degradative capacity of cells. Usually, these deposits are composed of ubiquitin conjugates, suggesting a failure in the clearance of proteins targeted for proteasomal degradation. The ubiquitin-proteasome system (UPS) is thought to be limited in its capacity to degrade aggregated proteins. Instead, their removal is believed to be primarily mediated by autophagy, a system by which cells sequester cytosolic cargo for delivery to lysosomes. The UPS and autophagy pathways were once considered separate systems but it now appears there is cross-talk and cooperation between them, with ubiquitin modification acting as a signalling molecule in both cases. In addition to its putative role in the targeting of ubiquitinylated cargo for proteasomal degradation, p62/SQSTM1 binds both target-associated ubiquitin and LC3 (an ubiquitin-like autophagy cascade protein) conjugated to the phagophore membrane, thereby effectively serving as an autophagic receptor for ubiquitinylated targets. Aggresomes, inclusion bodies that form when the ubiquitin–proteasome machinery is overwhelmed with aggregated proteins, may serve as storage depots for cargo awaiting degradation by autophagy. A cell-based assay was devised to detect protein cargo within aggresomes using a novel red fluorescent molecular rotor dye. Aggresomes were generated using various potent, cell permeable, proteasome inhibitors: MG-132, lactacystin, epoxomicin and bortezomib. Furthermore, amyloid beta peptide 1–42 was shown to induce aggresome formation in the SK-N-SH human neuroblastoma cell line. Co-localization of p62 and aggregated protein cargo within aggresomes was demonstrated by fluorescence microscopy. SMER28, a small molecule modulator of autophagy acting via an mTOR-independent mechanism, blocked accumulation of amyloid beta peptide within the cells. The described assay allows assessment of the effects of protein aggregation directly in cells, without resorting to the use of non-physiological protein mutations or genetically engineered cell lines.

BACKGROUND

In mammalian cells, aggregated proteins may be concentrated by microtubule-dependent retrograde transport to perinuclear sites of aggregate deposition, referred to as aggresomes. Aggresomes are inclusion bodies that form when the ubiquitin–proteasome machinery is overwhelmed with aggregation-prone proteins. Typically, an aggresome forms in response to some cellular stress, such as hyperthermia, viral infection or exposure to reactive oxygen species. Aggresomes appear to provide a cytoprotective function by sequestering the toxic, aggregated proteins and may also facilitate their ultimate elimination from cells by autophagy. Certain cellular inclusion bodies associated with human disease are thought to arise from an aggresomal response, including Lewy bodies associated with neurons in Parkinson's disease, Mallory bodies associated with liver cells in alcoholic liver disease and hyaline inclusion bodies associated with astrocytes in amyotrophic lateral sclerosis. We have developed a novel red fluorescent molecular rotor dye specifically devised to detect aggregated proteins within aggresome.

Introduction



Schematic depiction of autophagy. Cytosolic material is sequestered by an expanding membrane sac, the phagophore, resulting in the formation of a double-membrane vesicle, an autophagosome. The outer membrane of the autophagosome subsequently fuses with the lysosome, and the internal material is degraded in the autolysosome. Various regulators of autophagy are also depicted in the diagram. Aggresomes, inclusion bodies that form when the ubiquitin–proteasome machinery is overwhelmed with aggregated proteins, may serve as storage depots for cargo awaiting degradation by autophagy.

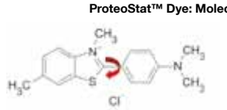
Classical Methods for Analyzing Aggresomes in Cells

Non-physiological protein mutations or genetically engineered cell lines have been developed for assessment of the effects of protein aggregation within cells.

Novel Approach

- Molecular rotor dye is used to highlight aggresomes
- Assay does not require non-physiological protein mutations or genetically engineered cell lines
- Suitable for fluorescence/confocal microscopy and high-content screening applications
- Cells are fixed, allowing for co-localization studies with labeled antibodies targeting various pathway proteins
- Assay is compatible with flow cytometry, allowing easy quantitation of cell response

ProteoStat™ Dye: Molecular Rotor Mechanism



Thioflavin T – Early prototype dye in the design of the Proteostat™ Aggresome assay. Enzo synthesized ~130 novel molecular rotor dyes and screened them for their ability to detect aggresomes.

Dye interaction with aggregated protein cargo within aggresomes.

Cell Aggresome Assay Protocols

Fluorescence Microscopy

1. Grow HeLa cells directly onto glass slides or polystyrene tissue culture plates until ~80% confluent.
2. Treat the cells with the compound of interest and negative control cells with vehicle.
3. Carefully wash the cells twice with 1x Assay Buffer.
4. Fix and permeabilize cells
5. Wash cells, and dispense ProteoStat™ reagent. Protect the samples from light & Incubate for 30 minutes at 37°C.
6. Wash the cells, apply a coverslip and observe them under a fluorescence/confocal microscope using Texas Red filters.

Flow Cytometry

1. Grow cells to log phase for flow cytometry analysis.
2. Treat the cells with the compound of interest and negative control cells with vehicle.
3. Carefully remove the supernatant.
4. Fix and permeabilize the cells.
5. Wash cells, gently resuspend the pellet in 500 µl ProteoStat™ detection reagent.
6. Protect the samples from light and incubate for 30 minutes at room temperature.
7. Samples should be analyzed via flow cytometry using a 488 nm laser with the FL 3 channel.

ProteoStat™ Dye Detects Protein Aggregate Accumulation within Aggresomes

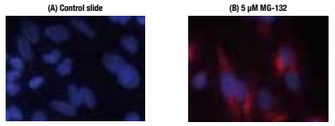


FIGURE 1: Cell Aggresome detected by ProteoStat™ dye as observed by fluorescence microscopy: Hela cells were mock-induced with 0.2% DMSO (A) or induced with 5 µM MG-132 (B) for 12 hours at 37°C. After treatment, cells were incubated with ProtepStat™ dye for 30 mins. This assay allows assessment of the effects of protein aggregation.

ProteoStat™ Dye Co-localizes with Fluorescein-p62 Ab

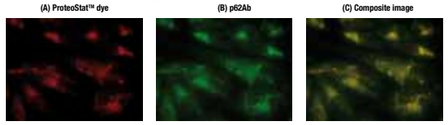


FIGURE 2: Cell Aggresome detected by ProteoStat™ dye co-localizes with fluorescein-p62 Ab, as observed by fluorescence microscopy: HeLa cells were treated for 12 hours with 5 µM MG-132 on a slide and stained with (A) ProteoStat dye and (B) Fluorescein-p 62 Ab and (C) composite image.

ProteoStat™ Dye Co-localizes with Fluorescein-LC3I/II Ab

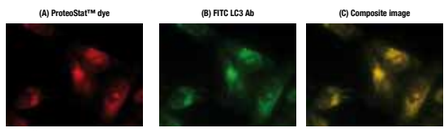


FIGURE 3: Cell Aggresome detected by ProteoStat™ dye co-localizes fluorescein-labelled antibody recognising LC3I/II, as observed by fluorescence microscopy: HeLa cells were treated for 12 hours with 5 µM MG-132 on a slide and stained with (A) ProteoStat dye and (B) Fluorescein-labelled antibody recognising LC3I/II and (C) composite image.

Assay Validation with Various Potent, Cell-Permeable & Selective Proteasome Inhibitors

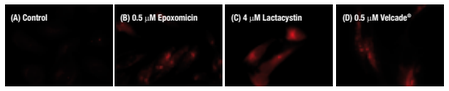
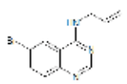


FIGURE 4: Cell Aggresome detected by ProteoStat™ dye was validated with various potent, cell permeable and selective proteasome inhibitors using Hela cells, as observed by fluorescence microscopy (A) control, (B) 0.5 µM Epoxomicin (C) 4 µM Lactacystin and (D) 0.5 µM Velcade® with overnight incubation.

Autophagy Modulator SMER28



SMER28 is a chemical inducer of autophagy which acts via an mTOR-independent mechanism. It increases autophagosome synthesis and enhances the clearance of model autophagy substrates such as A53T α-synuclein and mutant huntingtin fragments.

SMER28 attenuates mutant huntingtin-fragment toxicity in Huntington's disease cells and Drosophila models suggesting therapeutic potential [1,2].

References

[1] S. Sarkar et al. Nat. Chem. Biol. 2007 3 331

[2] M. Renna et al. J. Biol. Chem. 2010 285 11061

Amyloid Beta Peptide 1-42 Induces Aggresome Formation in the SK-N-SH Human Neuroblastoma Cell Line, which can be Blocked by SMER28

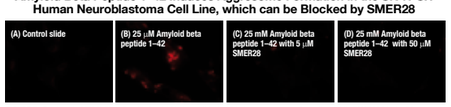


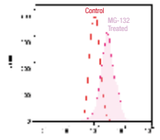
FIGURE 5: Treatment of SK-N-SH cells with Amyloid beta peptide 1–42 induces aggresome formation, while SMER28 blocks this accumulation, as demonstrated by fluorescence microscopy. (A) Untreated cells (B) 25 µM Amyloid beta peptide 1–42 M (C) 25 µM Amyloid beta peptide 1–42 with 5 µM SMER28 (D) 25 µM Amyloid beta peptide 1–42 with 50 µM SMER28.

Detection of Aggresomes with ProteoStat™ Dye Using Conditions Known to Modulate Autophagy and Proteasome Pathways

Treatment	Target	Effect	µM used	Induction Time (hrs)	Cell Line	Aggresome Formation
Starvation	Inhibits mammalian target of rapamycin (mTOR)	Activates autophagy	N/A	1–4	HeLa, HepG2, Jurkat	No
Rapamycin	Inhibits mammalian target of rapamycin (mTOR)	Activates autophagy	0.2	6–18	HeLa	No
PP242	ATP-competitive-inhibitor of mTOR	Activates autophagy	1	18	Hela	No
Lithium	Inhibits IMPase and reduce inositol and IP_3 levels; mTOR-independent	Activates autophagy	10,000	18	HeLa	No
Trehalose	Unknown, mTOR-independent	Activates autophagy	50,000	6	HeLa	No
Bafilomycin A1	Inhibits Vacuolar-ATPase	Inhibits autophagy	6–7^{10-1}	18	HeLa	Yes
Chloroquine	Alkalinizes Lysosomal pH	Inhibits autophagy	10–50	18	HeLa	Yes
Tamoxifen	Increases the intracellular level of ceramide and alcohlshes the inhibitory effect of PI3K	Activates autophagy	4–10	6–18	HeLa, HepG2, Jurkat	Yes
Verapamil	Ca^{2+} channel blocker; reduces intracytosolic Ca^{2+} levels; mTOR-independent	Activates autophagy	40-100	18	HeLa	Yes
Hydroxychloroquine	Alkalinizes Lysosomal pH	Inhibits autophagy	10	18	HeLa	Yes
Loperamide	Ca^{2+} channel blocker; reduces intra-cytosolic Ca^{2+} levels; mTOR-indepandant	Activates autophagy	5	18	HeLa	No
Clonidine	Imidazoline-1 receptor agonist; reduces cAMP levels; mTOR-independant	Activates autophagy	100	18	HeLa	No
MG-132	Selective proteasome inhibitor	Activates autophagy	4–10	18	HeLa, Jurkat	Yes
Norclomipramine	Alkalinizes Lysosomal pH	Inhibits autophagy	5–20	18	HeLa	Yes

TABLE 1: Treatments that influence autophagy, validated with ProteoStat™ dye.

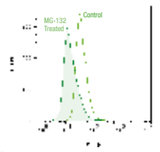
Identifying Protein Accumulation within Aggresomes by Flow Cytometry



Treatment	Mean (FL3) Signal
Control	113
Treated (5 µM MG132)	335
Aggresome Activity Factor	66

FIGURE 6: Flow cytometry-based analysis. Jurkat cells were mock-induced with 0.2% DMSO or induced with 5 µM MG-132 overnight at 37°C. After treatment, cells were fixed and incubated with ProteoStat™ dye, then analyzed by flow cytometry without washing using a 488 nm laser with fluorescence detection in the FL3 channel. Results are presented as histogram overlays. In MG-132 treated cells, fluorescent red signal increases about 3-fold. The described assay allows assessment of the effects of protein aggregation.

Identifying Protein Accumulation within Aggresomes with Fluorescein-p62 Ab by Flow Cytometry



Treatment	Mean (FL1) Signal
Control	17.8
Treated (5 µM MG132)	44
Aggresome Activity Factor	60

FIGURE 7: Flow cytometry-based analysis. Jurkat cells were mock-induced with 0.2% DMSO or induced with 5 µM MG-132 overnight at 37°C. After treatment, cells were fixed and incubated with Fluorescein-p62 Ab (1:500 dilution of stock), then after washing, analyzed by flow cytometry using a 488 nm laser with fluorescence detection in the FL1 channel. Results are presented as histogram overlays. In MG-132 treated cells, Fluorescein-p62 Ab signal increases about 2.5-fold.

Assessing Cytotoxicity Associated with Aggregated Protein Accumulation using Mito-ID® Membrane Potential Dye

The cationic carbocyanine potentiometric dye, Mito-ID® Membrane potential dye, is a dual emission probe that monitors energetic status.

Mito-ID® Membrane Potential Cytotoxicity Assay Protocols

Fluorescence Microscopy

1. Grow HeLa cells directly onto glass slides or polystyrene tissue culture plates until ~80% confluent.
2. Treat the cells with the compound of interest and negative control cells with vehicle.
3. Carefully wash the cells twice with 1x Assay Buffer and dispense the Mito-ID® Membrane potential dye in a volume sufficient for covering the cell monolayer.
4. Protect the samples from light and incubate for 15 minutes at room temperature.
5. Flick the staining solution onto a paper towel, apply a coverslip and observe under a fluorescence/confocal microscope: Use a rhodamine filter set for energized mitochondria; an FITC filter set for depolarized mitochondria.

Microplate Fluorescence Reader

1. Plate HeLa cells in a 96-well black wall/clear bottom plate and place in a 37°C humidified CO_2 incubator overnight.
2. Treat the cells with the compound of interest and negative control cells with vehicle.
3. Dispense 100 µl of the Mito-ID® Membrane potential detection reagent for each well. Protect samples from light and incubate for 15-30 minutes at room temperature.
4. Observe immediately with a fluorescent microplate reader using an excitation filter of about 480 nm and an emission filter of about 590 nm for the orange-fluorescent aggregated signal from the membrane potential reagent.

MG-132-Induced Aggresome Accumulation Leads to a Loss in Mitochondrial Membrane Potential

FIGURE 8: Hela cells were mock-induced with 0.2% DMSO (A) or induced with 2.5 µM MG-132, 5 µM MG-132 or 10 µM MG-132 overnight at 37°C. After treatment, cells were incubated with Mito-ID® Membrane potential dye, and visualized by epifluorescence microscopy.

Quantifying Mitochondrial Membrane Potential Loss in Cells Treated with MG-132

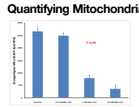


FIGURE 9: Using a conventional fluorescence microplate reader, mitochondrial membrane potential was shown to decrease as a function of MG-132 concentration. No wash and no medium removal is required for this assay. The high Z-factor (≥0.59) obtained using the Mito-ID® Membrane potential dye demonstrates excellent signal-to-noise ratios.

CONCLUSIONS

- ProteoStat™ dye provides a rapid, specific and quantitative approach for detecting denatured protein cargo within autophagosomes in fixed and permeabilized cells
- The described aggresome assay can be performed by fluorescence microscopy or by flow cytometry
- The assay was validated with small molecule modulators known to influence autophagy and proteosome pathways
- The assay is suitable for co-localization studies with fluorescently-labeled antibody conjugates, highlighting interactions between aggregated protein cargo and proteins implicated in aggresome formation, such as p62 and LC3
- The assay enables screening of aggregation inhibitors, relevant to neurodegenerative disease, in an authentic cellular context
- Parallel assays using Mito-ID® Membrane potential dye allow assessment of aggresome-induced cytotoxicity

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