A Novel Action of Histone Deacetylase Inhibitors in a Protein Aggresome Disease Model

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Supplemental Experimental Procedures

Adenovirus Production

Various SOD (wild-type, A4V, G93A, and G85R) full-length clones were generated by PCR using the following primers: 5 prime, 5′-GGGGGGAATTCTGATGGCGACGAAGG-3′; 3 prime, 5′-GACCGGT GGATCCTGGGCGATCCCAATTAC-3′.

These full-length cDNAs were then cloned into pShuttle CMV-EGFP-N1 (generous gift from J. Hoyt, ICCB) to generate in-frame SOD-GFP fusion. Adenoviruses expressing these fusion proteins were generated following the protocol by He et al. [S1].

High-Throughput Screen Protocol

To find small molecules that inhibit aggresome formation, we infected COS1 cells with Adenovirus expressing G85RSOD-GFP in clear-bottom 384-well plates. After incubation overnight at 37°C, small molecules were pin-transferred from a commercial library. The proteasome inhibitor ALLN (10 μg/ml) was added 4 hr post pin-transfer to induce aggresome formation. After 16 hr incubation, the cells were fixed and fluorescent images were captured by automated microscopy (http://iccb.med.harvard.edu/screening/technology_screen_by_imag/index.htm). These were scored by eye for enhancement or absence of aggresomes.

Small Molecule Libraries

The small molecules that are used in this study are from the following libraries: Chem Bridge Diverset E (16,320 compounds), NCI Structural Diversity Set, Version 1 (1,900 compounds), and NINDS Custom Collection (1,040 compounds). For more information, please go to http://iccb.med.harvard.edu/screening/compound_libraries/index.htm.

Cellular Fractionation and Immunoblotting

COS1 cells were fractionated according to Johnston et al. [S2] with minor modifications. Cells expressing mutant or wild-type SOD-GFP were washed once with ice-cold phosphate buffered saline (PBS), lysed with lysis buffer (10 mM Tris HCl [pH 7.5], 5 mM EDTA, 1% NP-40, 0.5% deoxycholate (DOC), 150 mM NaCl with 0.25 mM PMSF) on ice for 10 min. The lysate was passed through a 25-gauge needle 10 times on ice to break up the DNA. The insoluble fraction was collected at 16,000 × g for 30 min at 4°C. The soluble and the insoluble fractions were denatured with equal volume (250 μl) of SDS-PAGE sample buffer at 95°C for 5 min. The insoluble fraction was vortexed for 30 s. The heating-vortexing cycle was repeated three times to break up genomic DNA. One-tenth of total samples from soluble and insoluble fractions were loaded onto a 10% SDS-PAGE. The resultant electrophoregrams were blotted onto nitrocellulose membrane (Amersham Pharmacia). Immunoblot analysis used different antibodies and the enhanced chemiluminescence reagent (ECL-plus, Amersham Pharmacia).

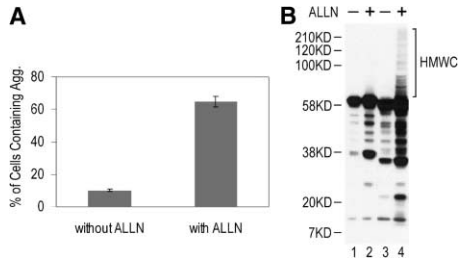

Figure S1. Percentage of COS1 Cells Containing Aggresomes Increase Significantly after Treatment with Proteasome Inhibitor ALLN

(A) COS1 cells were infected with adenovirus expressing G85RSOD-GFP. After overnight incubation at 37°C, the cells were treated with or without proteasome inhibitor ALLN at 10 μg/ml. After 16 hr incubation, cells were fixed, and the percentage of cells containing aggresomes (Agg) was calculated using an average of five independent experiments. Error bar stands for standard deviations (SDs). Student's t test, $p < 0.005$.

(B) COS1 cells were infected with adenovirus expressing WTSOD-GFP (lanes 1 and 2) or G85RSOD-GFP (lanes 3 and 4). After overnight incubation, the cells were treated with or without ALLN at 10 μg/ml. After 16 hr incubation, cells were lysed with 1× SDS-PAGE sample buffer. Equal amount (10%) of the total cell extracts were loaded in each lane. HMWC, high molecular weight complexes.

Supplemental References

S1. Kawaguchi, Y., Kovacs, J.J., McLaurin, A., Vance, J.M., Ito, A., and Yao, T.-P. (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell *115*, 727–738.

S2. Garcia-Mata, R., Bebok, Z., Sorscher, E.J., and Sztul, E.S. (1999). Characterization and dynamics of aggresome formation by a cytosolic GFP-chimera. J. Cell Biol. *146*, 1239–1254.

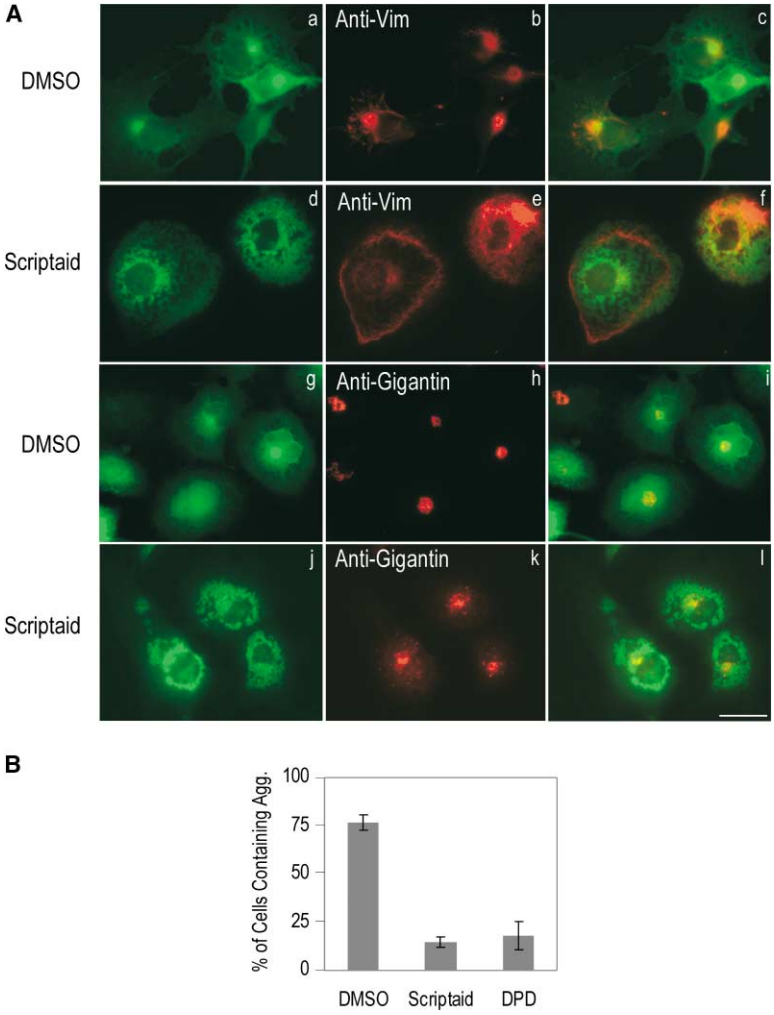

Figure S2. Scriptaid and DPDTB Prevent Vimentin Network from Collapsing and Do Not Affect Microtubule Organizing Center

(A) COS1 cells expressing G85R-GFP were treated with either DMSO (a–c and g–i) or Scriptaid (d–f and j–l) along with protease inhibitor (10 μg/ml ALLN) overnight. The cells were then fixed and stained with anti-vimentin (Hybridoma bank 40EC) (b and e) or anti-Gigantin (BD biosciences) (h and k) antibody using standard immunofluorescence technique. In cells with aggresomes, vimentin loses its filamentous staining pattern and collapses into a ring-like structure surrounding aggresomes (a–c). In cells treated with Scriptaid, short vimentin filaments localized near mSOD-GFP microaggregates but did not completely colocalize with these aggregates (d–f) nor collapse into perinuclear ring-like structure. A similar phenotype was observed with DPD treatment (data not shown). To check whether MTOCs are completely blocked, we examined the distribution of the Golgi network. In mSOD-GFP expressing cells, the Golgi network was slightly disrupted in that the Golgi structure localized around the aggresomes as shown by staining with the Golgi marker gigantin (g–i). This was consistent with previous observations on aggresomes formed by GFP-250. In the presence of Scriptaid, gigantin was mostly concentrated at the MTOC, with a slightly more dispersed distribution in treated than untreated cells (j–l). a, d, g, and j, G85R-GFP staining; c, f, i, and l, corresponding superimposed images. Scale bar equals 40 μm.

(B) The percentage of COS1 cells containing aggresomes decreases significantly after treatment with Scriptaid and DPD. COS1 cells infected with adenovirus expressing G85RSOD-GFP were treated with Scriptaid, and DPD at 5 μM along with DMSO 16 hr postinfection. 4 hr after adding compounds, proteasome inhibitor ALLN was added at 10 μg/ml. After overnight incubation, cells were fixed and the percentage of cells containing aggresomes (Agg.) was measured. The statistics were based on five independent experiments. Error bar, SDs. Student's t test, $p < 0.01$.

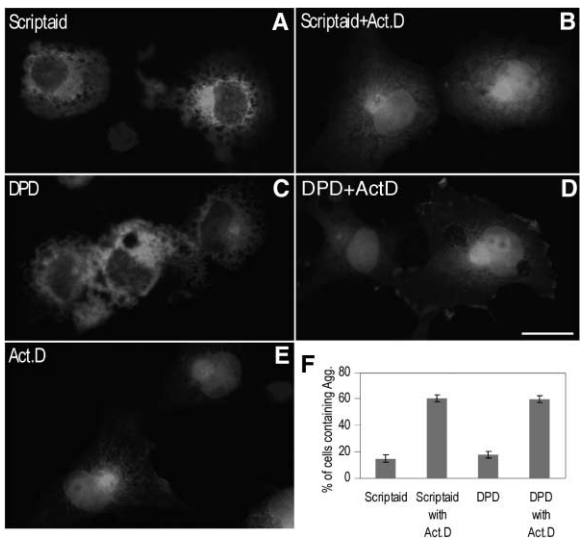

Figure S3. The Effect of Scriptaid and DPDTB on Aggresome Formation Is Dependent on Transcription

(A–E) COS1 cells expressing G85R-GFP were treated with Scriptaid (5 μM) or DPD (5 μM) for 4 hr before adding proteasome inhibitor (10 μg/ml ALLN) with or without Actinomycin D (5 μg/ml). The cells were fixed after overnight incubation at 37°C. Most cells did not form aggresomes when treated with Scriptaid or DPD with ALLN (A, C, and F). In the presence of Actinomycin D, the characteristic perinuclear structure reappeared in most of the cells (B, D, and F). Treatment with Actinomycin D along with ALLN still formed aggresomes in most cells (E).

(F) Statistical analysis of percentage of cells containing aggresomes under different treatment. The data was the summary of three independent experiments. Error bar, SDs. Student's t test, $p < 0.01$.