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

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Summary

Protein inclusions are associated with a number of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) [1]. Whether protein aggregates are toxic or beneficial to cells is not known. In ALS animal models, mutant SOD1 forms aggresome-like structures in motor neurons and astrocytes [2, 3]. To better understand the role of protein aggregation in the progression of disease etiology, we performed a screen for small molecules that disrupt aggresome formation in cultured cells. After screening 20,000 compounds, we obtained two groups of compounds that specifically prevented aggresome formation. One group consists mainly of cardiac glycosides and will be the subject of another study. The second group contains two compounds: one is a known histone deacetylase (HDAC) inhibitor, Scriptaid [4], and the other is a Flavin analog, DPD. Cells treated with these molecules still contained microaggregates, but these microaggregates were not transported to microtubule organizing centers (MTOCs). The defect in transport was linked to modulation of the dynein/dynactin machinery as treatment with Scriptaid or DPD reversed mSOD-induced insolubilization of the dynactin subunits P50 dynamitin and P150^{glued}. Our findings suggest a connection between HDAC activity and aggresome formation and also lay the groundwork for a direct test of the role of aggresome formation in ALS etiology.

Results and Discussion

Mutant SOD-GFP, but Not Wild-Type SOD-GFP, Forms Aggresomes in COS1 Cells

Mutations in the cytoplasmic metalloenzyme Cu/Zn superoxide dismutase 1 (SOD1) cause about 20% of familial ALS [1, 5]. Mutant SOD1 forms aggresome-like structures in patients, tissue culture cells, and animal models of ALS [2, 6–8]. To develop an assay for aggresome assembly for screening small molecules, we generated adenoviruses expressing mutant (A4V, G85R, and G93A) and wild-type (WT)SOD-GFP fusion proteins and expressed them in COS1 cells. WTSOD-GFP-expressing cells showed diffuse, uniform cytoplasmic fluorescence that was little affected by adding proteasome inhibitors. Cells expressing mSOD-GFP, in contrast, showed granular fluorescence, and some showed aggresome formation, evident from GFP staining concentrating at the microtubule organizing center (MTOC). When cells expressing mSOD-GFP were treated with proteasome inhibitors (10 μg/mlALLN), bright perinuclear structures characteristic of aggresomes formed in more than 65% of cells (Figure 1A and Figure S1A in the Supplemental Data available with this article online).

We believe the reason that mSOD-GFP accumulates in aggresomes is because mutations in SOD prevent proper folding, in turn promoting accumulation of unfolded mSOD protein. To test this, we fractionated COS1 cells expressing mSOD-GFP and WTSOD-GFP into detergent-soluble and -insoluble fractions. The fraction of fusion protein present in the insoluble fraction is markedly higher for mSOD-GFP than for WTSOD-GFP. Even in the absence of proteasome inhibitors, the insoluble mSOD-GFP protein forms high molecular weight complexes that are reactive to anti-SOD antibody and resistant to SDS denaturation. In the presence of the proteasome inhibitor ALLN, these complexes are formed much faster (Supplemental Figure S1B). WTSOD-GFP does not form similar complexes (Figure 1B).

These observations are very similar to those made previously on mSOD expressed in tissue culture cells [6]. In that study, the insoluble mSOD protein complex was found not to be ubiquitinated. However, when we immunoprecipitated the mSOD-GFP from the insoluble fraction using anti-SOD antibody, we were able to detect high molecular weight ladders using anti-ubiquitin antibody, suggesting that the high molecular weight complex formed by mSOD-GFP was ubiquitinated (Figure 1C). This finding is consistent with previous findings in animal models of ALS, where the protein inclusions found in affected motor neurons react strongly to ubiquitin antibody [7]. These data convinced us that mSOD-GFP is a reasonable model to study mSOD aggregation.

We concluded from these data that mSOD-GFP is less stable than WTSOD-GFP. It tends to precipitate and readily forms aggresomes in COS1 cells upon inhibition of the protein degradation machinery. We developed a high throughput assay based on this observation to screen for compounds that disrupt aggresome formation.

Several Groups of Compounds Inhibit Aggresome Formation

To find small molecules that inhibit aggresome formation, we developed a screening protocol using COS1 cells infected with adenovirus expressing G85RSOD-GFP (see Supplemental Data).

After screening 20,000 compounds, we identified 25 compounds that reproducibly inhibited aggresome formation. Subsequent testing allowed us to group the screening positives into three categories: microtubule inhibitors (9), protein synthesis inhibitors (4), and candidate-specific aggresome inhibitors (12) (Figure 2). Microtubule inhibitors were detected by examining micro-

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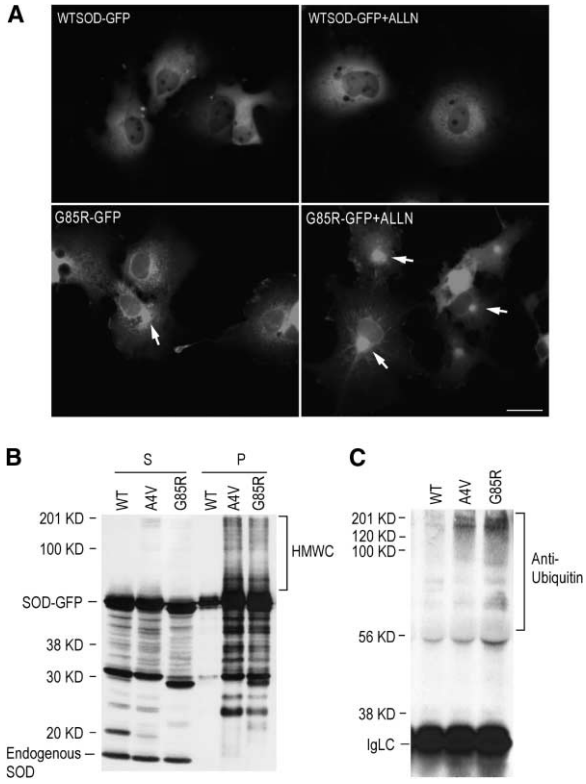

Figure 1. Mutant SOD (G85R)-GFP but not WTSOD-GFP Fusion Protein Forms Aggresomes upon Treatment with Proteasome Inhibitor ALLN

(A) Mutant SOD-GFP but not WTSOD-GFP fusion protein forms aggresomes. Fluorescence image of COS1 cells expressing G85R-GFP or WTSOD-GFP fusion protein incubated overnight with or without proteasome inhibitor (10 μg/ml ALLN). Scale bar equals 40 μm.

(B) Mutant but not WTSOD-GFP fusion protein forms high molecular weight complexes (HMWC) that are resistant to SDS denaturation. COS1 cells infected with Adenoviruses expressing mutant (G85R or A4V)-SOD or wild-type SOD-GFP fusion protein were incubated for 2 days without proteasome inhibitors. Total cell extracts were fractionated into detergent-soluble and -insoluble fractions according to the experimental procedure. Equal amounts (10%) of extract fractions were immunoblotted with anti-SOD antibody (Calbiochem #574597). S, soluble fraction; P, insoluble fraction.

(C) The high molecular weight complexes formed by mutant SOD-GFP are ubiquitinated. Total cell extracts from cells expressing either mutant (A4V or G85R)-GFP or wild-type SOD-GFP fusion proteins were immunoprecipitated using RIPA buffer with anti-SOD antibody according to the standard protocol [17]. The antibody bound fraction was immunoblotted with anti-ubiquitin antibody (Sigma U5379). Ig LC, IgG light chain.

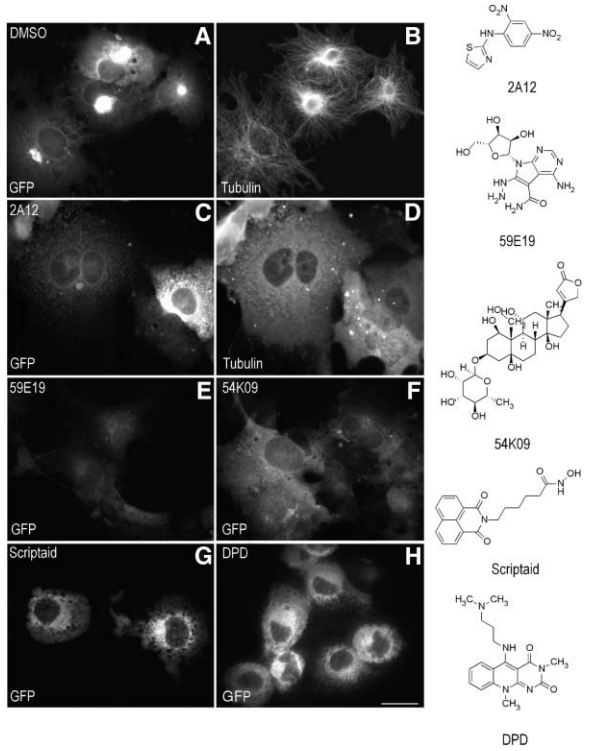

Figure 2. Structures of Hit Compounds and Their Corresponding Phenotype

(A) GFP staining pattern of DMSO control.

(B) α-tubulin morphology shown by immunofluorescence staining with anti-α-tubulin antibody (Sigma T5168) under DMSO treatment.

(C) GFP staining pattern of cells treated with 2A12, a microtubule destablizer.

(D) α-tubulin morphology with 2A12 treatment, labeled as in (B).

(E) Phenotype of protein synthesis inhibitor 59E19.

(F) Phenotype of Cardiac Glycoside 54K09.

(G) Phenotype of Scriptaid.

(H) Phenotype of DPD.

(A), (C), and (E)–(H) show GFP staining pattern. Scale bar equals 40 μm.

tubule structure in the presence of the small molecule. An example of this group, compound 2A12, is shown in Figures 2C and 2D. Protein synthesis inhibitors were identified by quantifying total GFP signal and ^{35}S methionine incorporation into total cellular proteins (data not shown). Compound 59E19 is an example of this group (Figure 2E). Only the specific inhibitors, those whose activity could not be explained by effects on microtubules or protein synthesis, were pursued further. These fell into two groups. The first group is composed of mainly cardiac glycosides (an example is shown in Figure 2F) and will be discussed elsewhere. The second contains two compounds. The first, 14N02, is a hydroxamic acid-based histone deacetylase (HDAC) inhibitor, named Scriptaid [4]. The second, DPD (5-(3-Dimethylamino-propylamino)-3,10-dimethyl-10*H*-pyrimidol[4,5-*b*]quinoline-2,4-dione) is a Flavin analog whose effects on cells are unknown. The two compounds have different structures but give a similar phenotype. The mutant SOD-GFP fusion protein is present in treated cells at similar or higher levels than control cells. mSOD-GFP forms small granular structures throughout the cytoplasm of treated cells, but assembly into aggresomes is almost completely inhibited in most cells (Figures 2G and 2H). The percentage of cells containing aggresomes decreases from 77% with DMSO to 15% with 5 μM of Scriptaid and 18% with 5 μM of DPD (Supplemental Figure S2B). We tested the potent HDAC inhibitors Trichostatin A and Trapoxin [9]. These have a similar effect at concentrations of 1 μM and 10 nM, respectively, but

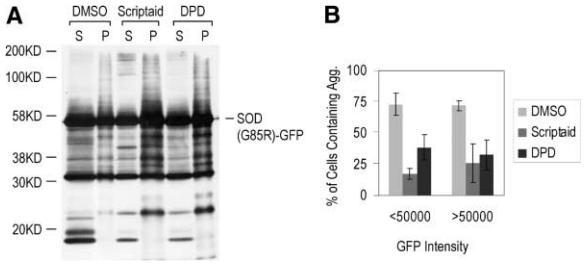

Figure 3. Scriptaid and DPD Do Not Block Microaggregation, and Their Effects Are Independent of Expression Level

(A) Neither Scriptaid nor DPD solubilized mutant SOD-GFP fusion protein. COS1 cells expressing G85R-GFP fusion protein were treated with Scriptaid, DPD, or control DMSO overnight. Cells were then fractionated into detergent-soluble (S) and -insoluble (P) fractions according to experimental procedure. The amount of G85R-GFP protein in each fraction was examined by immunoblotting with anti-SOD antibody. S, soluble fraction; P, insoluble fraction.

(B) The percentage of cells containing aggresomes is similar at different protein expression levels. Protein expression level was measured by GFP intensity (arbitrary units) in three independent experiments. Error bars represent standard deviations (SDs). The differences between treated and untreated are significant according to Student's t test. $p < 0.005$.

are more toxic to cells (data not shown). We therefore concluded that HDAC inhibitors inhibit aggresome formation in our assay.

Scriptaid and DPD Do Not Block Microaggregation, but Affect the Interaction of Misfolded Mutant SOD-GFP Protein with Dynein/Dynactin Machinery

To understand how Scriptaid and DPD inhibit aggresome formation, we examined their effect on the distribution of the aggresome markers, including vimentin and gigantin [10, 11] (see Supplemental Figure S2). In cells treated with Scriptaid or DPD, vimentin does not form the characteristic ring-like structure found in aggresome-containing cells (Supplemental Figure S2A, a–f) [10, 11]. The close to normal localization of vimentin confirms that Scriptaid and DPD block aggresome formation. Additionally, gigantin was mostly concentrated at the MTOC with a slightly more dispersed distribution in treated than untreated cells (Supplemental Figure S2A, g–l). In a nocodazole washout experiment, the assembly of the Golgi apparatus was not affected (data not shown) in the presence of these molecules. These data suggest that Scriptaid and DPD do not greatly affect MTOC formation or Golgi localization and assembly.

One possible explanation is that our compounds prevent aggresome function by binding to and solubilizing misfolded proteins. To test this, we fractionated cells expressing mSOD-GFP into detergent-soluble and -insoluble fractions. In untreated cells, about 55% of fusion protein is detergent insoluble. Treatment with Scriptaid and DPD does not change this fraction (Figure 3A). This indicates that mSOD-GFP still forms protein aggregates in the presence of these molecules, consistent with the appearance of dispersed microaggregates by the GFP image.

In cells treated with Scriptaid or DPD, the level of mSOD-GFP is enhanced compared with that in untreated cells (Figures 2G and 2H), presumably due to enhanced transcription from the CMV promoter. We considered the possibility that the effect of these compounds is due to enhanced expression of SOD-GFP overwhelming the aggresome assembly pathway. To test this, we compared the percentage of cells containing aggresomes at different protein expression levels. Total GFP signal per cell was measured and the cells were assigned to a high or low expression group using an arbitrary cutoff. The fraction of cells assigned to the high expression groups was higher in the presence of Scriptaid (62%) and DPD (53%) than DMSO (37%), as expected. However, expression level did not correlate with aggresome assembly. The percentage of cells containing discrete aggresomes is determined by counting 300 cells randomly for each group. Figure 3B shows that about 73% of control cells contain aggresomes regardless of the protein expression level. The percentage was reduced to no more than 25% and 37% for treatment with Scriptaid and DPD, respectively (Figure 3B). Since aggresome assembly was similar in high and low expressors, we concluded that the effect could not simply be due to the enhanced expression of SOD-GFP protein.

The effect of Scriptaid and DPD is similar to microtubule destabilizers such as 2A12 and nocodazole in that mutant protein forms microaggregates that are not transported to MTOCs. Although the microtubule distribution is not apparently affected and expression levels of dynein/dynactin appear normal, it is possible that inhibition of aggresome assembly by Scriptaid and DPD might be due to some other effect on dynein/dynactin transport. To test this, we measured the fraction of transport proteins that were recruited to insoluble components by cell fractionation (Figure 4). Cells were lysed with detergent, separated into soluble and insoluble components (see Supplemental Experimental Procedures), and probed with antibodies to dynein/dynactin components. In cells expressing mSOD-GFP, there was an increase in the amount of detergent-insoluble $P150^{glued}$ and P50 dynamitin compared with cells expressing WTSOD-GFP, consistent with the idea that these proteins are recruited to insoluble mSOD-GFP aggregates. There was also a change of solubility for protein chaperone Hsp70 but not Hsp90 (Figure 4A). This latter observation is similar to previous studies showing that a portion of Hsp70 becomes detergent insoluble in NIH 3T3 cells expressing a mutant form of SOD [12]. When cells expressing mSOD-GFP were treated with Scriptaid or DPD, the amount of insoluble P150 decreased from 30% to 11% and 16%, respectively (Figures 4B and 4C), and the amount of insoluble P50 dynamitin subunit decreased significantly from 20% to less than 5% (Figures 4B and 4C). The solubility of α-tubulin, a control protein, was not affected by any treatment. The amount of insoluble Hsp70 did not change with compound treatment (data not shown). These data suggest that our compounds may interfere with the interaction of the mSOD-GFP aggregates with the dynein/dynactin machinery, explaining the lack of transport to the MTOC.

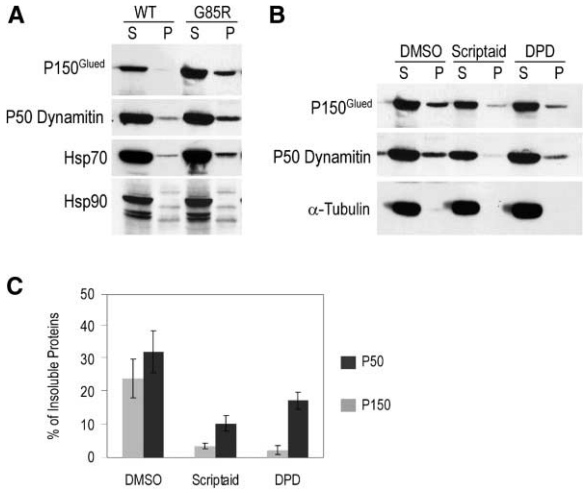

Figure 4. Both Scriptaid and DPD Promote the Solubilization of P150^{glued} and P50 Dynamitin

(A) Like heat shock protein Hsp70, P150^{glued} and P50 dynamitin show increases in the fraction of protein that is soluble in cells expressing mSOD-GFP. COS1 cells expressing either wild-type or G85R-GFP fusion protein were fractionated into detergent-soluble and -insoluble fractions as described in Supplemental Experimental Procedures. The amount of each protein was measured by immunoblotting using anti-Hsp70 (Sigma H5147), anti-Hsp90 (Sigma H1775), anti-P50 dynamitin (Pharmingen 611002), or anti-P150^{glued} (Pharmingen 610473).

(B) Treatment with Scriptaid and DPD decreased the amount of insoluble P150^{glued} and P50 dynamitin. COS1 cells expressing mutant G85R-GFP were treated with Scriptaid, DPD, and control DMSO. Total proteins were fractionated into detergent-soluble (S) and -insoluble (P) fractions according to the experimental procedure. The amount of P50 dynamitin, P150^{glued}, and tubulin were determined by immunoblotting.

(C) The percentage of insoluble P150^{glued} and P50 dynamitin decreased in the presence of Scriptaid and DPD. The amount of proteins in the soluble and insoluble fractions was calculated using NIH imaging software and the results were an average of four independent experiments. Error bar, SDs. The difference between each pair of data is significant according to Student's t test, for P50 dynamitin, $p < 0.02$, for P150^{glued}, $p < 0.03$.

In response to various physiological stresses, including heat shock or exposure to toxic agents, cells upregulate a conserved set of heat shock proteins via transcriptional regulation of the corresponding genes [13]. Misfolded proteins are competent to mediate a subset of the responses to heat shock in *Saccharomyces cerevisiae* [14]. Previous studies showed that in NIH 3T3 cells stably expressing a mutant form of SOD, the level of Hsp70 is upregulated [12]. We did not see any significant change in the level of Hsp70 and Hsp90 proteins. However, we did notice that expression of mSOD-GFP causes a portion of Hsp70 to become insoluble. Part of the discrepancy between our data and those of Shinder et al. [12] could be due to the fact that they used stably transfected cells and we used transient transfection with adenovirus. Interestingly, we also found that a portion of dynactin subunits become insoluble upon mutant SOD-GFP expression. It is possible that the dynactin complex binds to misfolded proteins or that it recognizes chaperones or ubiquitin conjugates. Very little is known about how misfolded proteins are recognized by cellular machinery and how they are recognized by the dynactin complex.

Deacetylase enzymes can be either cytoplasmic (e.g., tubulin deacetylase) or nuclear (e.g., histone deacetylase). We found that both Scriptaid and Trichostatin A inhibited deacetylation of both histones (H3, H4) and tubulin, indicating that they are a relatively broad inhibitors of deacetylases, but DPD did not, indicating that it is probably not an HDAC inhibitor (data not shown). A tubulin-specific deacetylase inhibitor Tubacin [15] had no effect at concentrations that are selective for tubulin acetylation (data not shown). These data suggest that the effect of Scriptaid might require inhibition of histone deacetylation, consistent with this notion; the effect of Scriptaid and DPD required transcription (Supplemental Figure S3).

We hypothesize that Scriptaid and DPD act through a transcription program that changes the expression profile of some protein factor(s) that are involved in the recognition and binding of protein aggregates by the dynactin complex. Since recognition of aggregates is poorly understood, it is difficult at this point to suggest candidate(s) for this induced protein(s). mRNA expression analysis might reveal candidates, and for such an approach, our identification of two molecules that modulate a transcription program by possibly different mechanisms may be useful.

It is known from previous studies that formation of aggresomes in cells does not affect trafficking between ER and the Golgi apparatus in tissue culture cells [11]. We show here that aggresome formation does cause some dynactin complex to become insoluble and thus, presumably, sequestered from the normal transport function. This could be damaging to neurons overexpressing high levels of misfolded proteins. Both Scriptaid and DPD promoted the solubilization of dynactin subunits in cells expressing mSOD. If sequestration of dynactin by aggregation causes toxicity, treatment of cells with these molecules might be beneficial to neurons containing aggresomes. Experiments are currently underway to test these compounds in ALS transgenic mice.

While this manuscript was under review, a study used genetics to show that the catalytic activity of histone deacetylase HDAC6 was required for aggresome formation [16]. In this study, the authors provided evidence that HDAC6 binds directly to both misfolded protein and the dynein/dynactin transport machinery. Our results are consistent with their conclusion, and in addition, this study provides small molecule tools for further dissecting the aggresome pathway.

Supplemental Data

Supplemental data including experimental procedures can be found online at http://www.current-biology.com/cgi/content/full/14/6/488/DC1.

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