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Biologically active molecules that reduce polyglutamine aggregation and toxicity

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Polyglutamine expansion in certain proteins causes neurodegeneration in inherited disorders such as Huntington disease and X-linked spinobulbar muscular atrophy. Polyglutamine tracts promote protein aggregation in vitro and in vivo with a strict length-dependence that strongly implicates alternative protein folding and/or aggregation as a proximal cause of cellular toxicity and neurodegeneration. We used an intracellular polyglutamine protein aggregation assay based on fluorescence resonance energy transfer (FRET) to identify inhibitors of androgen receptor (AR) aggregation in three libraries of biologically active small molecules: the Annotated Compound Library, the NINDS Custom Collection and a kinase inhibitor collection. In the primary screen 10 compounds reduced AR aggregation. While 10/10 also reduced huntingtin (Htt) exon 1 aggregation, only 2/10 reduced aggregation of pure polyglutamine peptides. In a PC-12 model 9/10 compounds reduced aggregation. Five out of nine compounds tested in an Htt exon 1 assay of neurodegeneration in Drosophila partially rescued the phenotype. Three of the five compounds effective in flies are FDA-approved drugs. These compounds provide new leads for therapeutic development for the polyglutamine diseases based on their efficacy in mammalian cells and a Drosophila model. The high predictive value of the primary screen suggests that the FRET-based screening assay may be useful for further primary and secondary screens for genes or small molecules that inhibit polyglutamine protein aggregation.

INTRODUCTION

Polyglutamine neurodegenerative diseases are devastating, but currently have no treatment. X-linked spinobulbar muscular atrophy (SBMA) is a progressive motor neuron disease caused by polyglutamine expansion in the N-terminus of the androgen receptor (AR) (1–3). Huntington disease (HD) is a progressive neuropsychiatric degenerative illness that is caused by polyglutamine expansion in the N-terminus of a protein of unknown function, huntingtin (Htt) (4). Enlarged glutamine tracts confer a toxic gain-of-function that manifests primarily in CNS neurons. This neurotoxic property is associated with a propensity for the expanded protein to misfold, aggregate and accumulate in intracellular inclusions. Polyglutamine misfolding and/or the aggregation process is likely central to pathogenesis, whereas formation of large inclusions is more controversial, and may play a role in pathogenesis, may be an epiphenomenon, or may even be protective (5–12).

Although expanded polyglutamine peptides spontaneously aggregate in vitro, much evidence suggests that aggregation and inclusion formation are highly regulated processes within the cell. For example, aggregation of polyglutamine proteins is modulated by the glucocorticoid receptor, the active form of MEKK1, cell stress and/or heat shock, the molecular chaperones (especially hsp70/40), the ubiquitin–proteasome system, JNK activation and Akt signaling (12–18). Although some of these regulatory pathways are likely part of the cellular misfolded protein/degradation responses (e.g. ubiquitin–proteasome, hsp70/40), others may uniquely inhibit polyglutamine protein aggregation. If so, pharmacologic or genetic manipulation of such pathways could be an effective therapeutic strategy.

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High-throughput screening (HTS) has previously been used to identify a limited number of small molecules that directly inhibit polyglutamine aggregation and/or toxicity (19–25). Alternatively, candidate-based approaches have identified potential therapeutic compounds such as histone deacetylase inhibitors, bile acids, cystamine, Congo red and its derivatives, anti-oxidants and caspase inhibitors, which have shown modest efficacy in vivo (26–34). The relative utility of various screening systems has not been rigorously analyzed, and thus further work is needed to determine the strengths and limitations of each.

When polyglutamine proteins are fused to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), FRET results from protein self-association, and is readily detected in large intracellular inclusions (35,36). FRET occurs when an energy donor, e.g. CFP, transfers energy to an acceptor molecule, e.g. YFP. FRET decreases rapidly when intermolecular distance exceeds ~80–100Å. We have previously developed a HTS system using FRET, and based on testing a selected group of compounds we identified a small molecule, Y-27632, that reduces polyglutamine aggregation in cell models, and toxicity in Drosophila (35). This validated the cell-based approach as a viable method to identify potential therapeutics for polyglutamine disease, but left uncertain its overall value for identifying compounds with activity in different cell and animal models.

Consequently, we have tested whether multiple small molecules that target cellular pathways to reduce AR and Htt aggregation in cell culture would reduce toxicity in vivo. We identified small molecule inhibitors of AR and Htt aggregation and toxicity by screening three independent libraries, comprising over 4000 biologically active small molecules of diverse structure. We identified 10 compounds that inhibited AR aggregation. These were further characterized to determine their effective concentrations, and their efficacy against aggregation of Htt exon 1 and a polyglutamine peptide using the FRET assay. In addition, we used detergent fractionation to test whether the compounds would inhibit Htt exon 1 aggregation in an inducible PC-12 model. Where possible, we tested related compounds to confirm structure/activity relationships. Finally, we tested 9/10 compounds in an established Drosophila model of neurodegeneration mediated by Htt exon 1.

RESULTS
Detection of aggregates via FRET
N-terminal polyglutamine containing fragments of AR and Htt, or pure polyglutamine stretches, are fused at their C-termini either to CFP or YFP (Fig. 1A). They are expressed in transiently transfected HEK293 cells, and cultured in 96-well dishes in the presence of the test compounds. After ~24 h, the cells are fixed and FRET is measured on a fluorescence plate reader (FPR) (35). To further characterize the FRET assay, we determined the predominant type of polyglutamine aggregates present in our cell culture system by measuring small detergent-extractable versus large detergent-insoluble species. First, we transfected HEK293 cells with unexpanded or expanded polyglutamine N-terminal truncation products of AR consisting of the first 127 amino acids fused to CFP and YFP [AR(Q25/65)CFP/YFP] (Fig. 1A). Next, we prepared lysates in the presence of 1% Triton X-100. We observed no significant FRET from AR(Q25)CFP/YFP in the lysate or the supernatant from a 14 000 g centrifugation step, consistent with the absence of aggregates. Conversely, lysate from cells expressing AR(Q65)CFP/YFP demonstrated FRET, and this signal was precipitated by both high- and low-speed centrifugation (14 000 or 1000g), indicating that it was likely to be derived from large, non-ionic detergent-insoluble species (Fig. 1B).

![Figure 1. FRET assay detects detergent-soluble and detergent-insoluble polyglutamine aggregation.](https://example.com/figure1.png)
We tested whether the plate reader was missing small aggregates in solution using size exclusion chromatography to compare AR(Q25)YFP and AR(Q65)YFP proteins from HEK293 cell lysates. After a 14 000g centrifugation step, we detected predominantly monomers in the supernatant (Fig. 1C), similar to previous observations (11). This indicated that small aggregates, or oligomers, are unlikely to constitute a significant source of FRET signal in this cell system, and thus the predominant type of polyglutamine aggregates are large, detergent-insoluble species.

Selection of compounds for HTS

HTS using random chemical libraries have certain advantages, but the interpretation of results in a cell-based assay is hampered by uncertainty about molecular targets. Consequently, to limit our discoveries to compounds with relatively well-understood mechanisms of action, and possibly to identify unique cellular mechanisms that regulate polyglutamine misfolding, we screened three distinct collections comprising over 4000 biologically active small molecules: (i) the Annotated Compound Library (ACL) of biologically active small molecules of diverse structure (~2800 compounds); (ii) a kinase inhibitor collection (~300 compounds kindly provided by Kevan Shokat, Ph.D., UCSF); (iii) the NINDS Custom Collection of FDA-approved drugs and natural products (~1040 compounds). The compounds in the three libraries were tested at two concentrations, 10 and 3 μM, in four replicates on the same plate. Dose-response studies were performed using commercially available compounds. Of the 4140 compounds screened, 10 final hits were obtained.

In each case, our EC50 determinations were consistent with published values for efficacy in vitro or in cell-based assays, where these were available. The average real-time Z-value for the assay was 0.65 for all plates (Supplementary Material, Table S2).

Hits from the cellular screen

Table 1 summarizes the results of the screen, Figure 3 indicates the structures of validated hits, and Figure 4 indicates dose–response data. Brief descriptions of the hits are listed below. P-values for all dose-response studies and tests of compounds against Htt exon 1 and polyglutamine peptide aggregation are listed in Supplementary Material, Table S2. All effects were statistically significant to at least P ≤ 0.005.

Ac-YVAD-cmk (29% Suppression) This compound specifically inhibits caspase 1 and shows limited caspase 5 inhibition. To confirm its target, we tested another peptide inhibitor of caspase 1, Ac-WEHD-fmk, which also inhibited AR aggregation. Thus, caspase-1, which has previously been implicated in the cell death processes in HD (33), may also participate in the regulation of polyglutamine misfolding.

Epidermal growth factor receptor tyrosine kinase inhibitors (27–30% Suppression) Three compounds were identified. Gefitinib and the two other tyrosine kinase inhibitors obtained from the kinase inhibitor library putatively target the kinase domain of the epidermal growth factor receptor (EGFR) (37,38). Based on our results, we tested a fourth EGFR tyrosine kinase inhibitor, tyrphostin AG1478, which also reduced aggregation by 25% (data not shown), highlighting...
HEK293 cells were co-transfected in 10 cm plates with AR(Q65)CFP/YFP, and a mutant form of the glucocorticoid receptor (GR
D) that promotes nuclear inclusion formation. The next day,
cells were counted and plated with 100 nM dex in duplicate 96-well daughter plates in wells containing single aliquots of compounds from the libraries of daughter plates. After 24 h, the cells
were fixed using 4% paraformaldehyde and washed with PBS. CFP, YFP and FRET measurements were then performed. Hits from the primary screen were tested with detailed dose-response studies, eliminating 90%. Ten lead compounds were obtained. Three hits were obtained from the ACL, three hits were obtained from the kinase inhibitor library (all putative EGFR inhibitors) and four hits were obtained from the NINDS library. Hits were tested against htt exon 1-CFP/YFP, and polyglutamine-CFP/YFP aggregation. All inhibited aggregation of AR and Htt exon 1. These results suggested that there might be common mechanisms by which the cell regulates AR and Htt protein aggregation/disaggregation, whereas such mechanisms might
the potential importance of this target in modulating the aggregation process.

Fosfosal (21% Suppression) This is a non-acetylated salicylic acid derivative, used as a non-steroidal anti-inflammatory compound. The mechanism of action of this drug is not well-understood.

Levonordefrin (21% Suppression) This is an α-adrenergic agonist and is used as an intradermal vasoconstrictor in conjunction with local anesthetics.

Molsidomine (20% Suppression) This is a nitric oxide-releasing prodrug, which is used to treat myocardial ischemia. Liver esterases convert molsidomine to the active metabolite SIN-1, which then releases NO (39).

Nadolol (19% Suppression) Nadolol is an orally available, non-selective β-adrenergic receptor antagonist. We also tested a more selective β₁ antagonist (atenolol), and a more selective β₂ antagonist (butoxamine). However, neither reduced aggregation, suggesting that the effects of nadolol might be off-target, or that inhibition requires simultaneous β₁ and β₂ blockade.

Piceatannol (23% Suppression) This compound inhibits syk kinase, and was also recently identified in a screen of ataxin-1 phosphorylation inhibitors (25). Syk is a cytoplasmic protein that consists of two N-terminal Src homology domains in tandem, and is activated either by the autophosphorylation of these domains or by transphosphorylation by a Src family kinase (40). Syk kinase is involved in intracellular signal transduction, and may influence phosphatidylinositide 3 kinase signaling upstream of Rac (41-43).

Todralazine (19% Suppression) This compound has been employed as a smooth muscle relaxant. Its mechanism of action is not well understood. We also tested a structurally related FDA-approved compound, hydralazine, which is used as a vasodilator. Hydralazine reduced AR aggregation by 21% (data not shown). Because it is FDA-approved, we tested hydralazine in the Drosophila model.

Selectivity of hits for AR and Htt versus polyglutamine fusions to CFP/YFP

To assess the generality of the pharmacological effects, we next tested hits against Htt exon 1-CFP/YFP and polyglutamine-CFP/YFP aggregation (Table 1). Without exception, compounds effective against AR aggregation had similar activity against Htt exon 1. However, only 2/10 compounds showed any significant effect on polyglutamine-CFP/ YFP peptide aggregation (Table 1). This is unlikely to be due to increased aggregation kinetics of the polyglutamine peptide, because the FRET assay indicated that relatively less aggregation was occurring for this construct than Htt exon 1. These results suggested that there might be common mechanisms by which the cell regulates AR and Htt protein aggregation/disaggregation, whereas such mechanisms might
not necessarily apply to polyglutamine peptides that lack appropriate flanking sequences.

**Efficacy of compounds in an inducible PC-12 model**

To test whether compounds we identified might have efficacy in a distinct neural cell system, we independently evaluated them in an inducible PC-12 model of Htt exon 1 aggregation. In this model, a PC-12 cell line is used in which the ecdysone receptor (EcR) is stably expressed. Htt exon 1 (Q92) is cloned downstream of an EcR-inducible promoter. Addition of 1 μM ponasterone, an EcR agonist, induces expression of Htt exon 1, which forms intracellular inclusions (34). We cultured these cells for 48 h with 1 μM ponasterone in the presence of test compounds at 10 μM, or with Y-27632 as a positive control. At this point the cells were harvested and lysed in buffer containing 1% Triton X-100 in PBS. After 14,000g centrifugation, equivalent amounts of supernatant or pellet fractions were dissolved in SDS-sample buffer and resolved by SDS–PAGE, followed by western blot using anti-GFP antibody. Both supernatant and pellet fractions were evaluated and quantified by digital imaging. To control for levels of protein expression, the ratio of signal between the pellet and supernatant was compared for each condition, and normalized between each experiment, with unity representing the ratio of pellet/supernatant in the untreated sample. This experiment was repeated three times. All compounds except todralazine were effective at reducing the amount of Htt exon 1-GFP present in the pellet fraction in a manner comparable to the positive control compound, Y-27632 (Fig. 5).

**Efficacy of compounds in Drosophila**

We tested 9/10 of the hits for efficacy in a Drosophila model of neurodegeneration that is based on using the Gal4/UAS system to express in the nervous system a pathogenic fragment of mutant human Htt exon 1, and which has correctly predicted a class of compounds (HDAC inhibitors) with efficacy in mouse models (26,44–46). One molecule (EGFR kinase inhibitor no. 4) was not available for these tests, as it has been synthesized only in small quantities. Flies harboring a UAS:Htt exon 1 (Q93) gene were crossed to those harboring elav:Gal4, resulting in pan-neuronal expression of Htt exon 1. This causes progressive degeneration of many neuronal structures, including the photoreceptor neurons, which can be conveniently quantified using the pseudopupil technique (26,47). Adult (post-eclosion) Elav:Gal4; UAS:Htt exon 1 (Q93) flies were grown for 7 days on food containing test compounds at 10-fold dilutions (0.5, 5, 50 μM). Five of the nine compounds showed efficacy (Fig. 6). This indicates a relatively high predictive value for the cellular aggregation assay to ident-
ify small molecules that reduce neurodegeneration in Drosophila.

DISCUSSION

We have used a FRET-based cellular polyglutamine aggregation assay to screen three libraries of biologically active small molecules: the ACL, a collection of kinase inhibitors and the NINDS Compound Collection. Compounds were screened in duplicate for suppression of AR aggregation. Hits were validated by determining dose responses, and, where possible, by alternative pharmacologic inhibition. Hits from this primary assay were also evaluated using the FRET assay for inhibition of Htt exon 1-CFP/YFP and polyglutamine-CFP/YFP fusion protein aggregation. We observed perfect concordance for all 10 hits between AR and Htt exon 1, although only 2/10 compounds showed efficacy against the polyglutamine peptide. In an independent cellular analysis, we tested each of the hits for their ability to reduce detergent-insoluble material in an inducible Htt exon 1-GFP PC-12 cell line, where 9/10 compounds were effective. Finally, we tested 9/10 hits in a Drosophila assay of Htt exon 1-induced neurodegeneration. In this secondary assay, 5/9 compounds were effective, and three of these are FDA-approved drugs. This work advances our understanding of polyglutamine pathogenesis and the development of novel therapies in several ways. It has demonstrated that the FRET-based cellular assay has high predictive value for determining effects in secondary screens, which implies that it measures an important aspect of polyglutamine toxicity. This work has also confirmed that the regulation of polyglutamine aggregation is protein context-dependent, and not solely determined by the polyglutamine repeat, whereas there was perfect concordance for all hits in reducing AR and Htt exon 1 aggregation, only 2/10 compounds were effective against a polyglutamine tract fused to CFP/YFP. Finally, we have identified several lead compounds, some of which suggest signaling pathways (EGFR, syk kinase, caspase-1) that may be involved in regulating polyglutamine pathogenesis, and which may be fruitful avenues for future investigations.
There are relatively few published screening efforts for polyglutamine disease. Prior screens of the NINDS Compound Collection have yielded hits distinct from those we identified. Piccioni et al. (21), using polyglutamine-induced caspase-3 activation as a readout, identified certain cardiac glycosides and a calcium channel blocker; Aiken et al. (23), using polyglutamine-induced cell death as a readout, identified multiple compounds; Wang et al. (19), using an in vitro aggregation assay, identified two compounds, juglone and celastrol, which reversed an abnormal cellular phenotype in a full-length Htt striatal cell model. It is likely that the absence of concordance derives from the profoundly different screening criteria used in each case, and it will be informative to test compounds identified in these assays in various animal models to allow comparisons between the assays and to prioritize the compounds for further study.

Here we have exploited a highly sensitive and quantitative assay to probe for molecular modifiers of aggregation, and have subsequently tested their effects on toxicity in vivo. To increase the likelihood that hits from our screen could be moved more rapidly to the clinic, we consciously avoided high-throughput approaches based on large chemical libraries such as those previously tested against Htt aggregation in solution (24) and in yeast (22), because many of the compounds identified in these approaches may have limiting toxicities, or function via cellular mechanisms that are difficult to determine (which impedes optimization and detailed characterization). Effective hits in our system must operate within the context of mammalian cells grown over a period of 24–48 h. Because we are measuring a decrease in FRET signal with preservation of absolute levels of CFP and YFP, we readily eliminated all overly toxic compounds. The constituents of the libraries we tested (biologically active small molecules) also raised the likelihood that many hits would function via alteration of definable cellular mechanisms, which might make them more favorable for drug development. Our present results have not allowed determination of the precise mechanisms of action of the hits; indeed, we cannot rule out that they function by direct interaction with the expanded polyglutamine proteins. However, in several cases (caspase 1 inhibitor, EGFR inhibitors, todralazine), it was possible to pick similar compounds that showed equivalent, if not superior efficacy, based on predicted structure/function relationships. This further underscores the validity of these compounds (and their associated pathways) as therapeutic targets. In future work we will pursue mechanisms of action, and will test the leads in other Drosophila and mouse models of neurodegeneration.

Figure 6. Analysis of hits in a Drosophila model of neurodegeneration. Hits derived from the cellular assay were tested in the Drosophila model. Flies harboring a UAS:Htt exon 1 (Q93) gene were crossed to those harboring elav:Gal4, which results in pan-neuronal expression of Htt exon 1. This produces progressive degeneration of light-sensing rhabdomeres, which is quantified via the pseudopupil technique. Elav:Gal4; UAS:Htt exon 1 (Q93) flies were grown for 7 days after eclosion on food containing test compounds at 10-fold dilutions (0.5, 5, 50 \( \mu \)M). (A–E) Dose responses of compounds that showed efficacy. Analysis by average number of rhabdomeres/ommatidium per fly demonstrated a significant reduction in rhabdomere loss at 7 days, representing reduction in degeneration. Error bars represent the SEM. (F) Summary of all compounds tested in Drosophila, with their relative rescue of toxicity.

AR and Htt aggregation: similar mechanisms of cellular regulation

As pure polyglutamine stretches in many respects recapitulate the misfolding of peptide fragments derived from native proteins, and have been used in vitro and in cell models to study pathology (48–50), one might assume that the aggregation/disaggregation of polyglutamine peptides would be regulated similar to that of AR and Htt exon 1. This may be an oversimplification. We observed complete concordance between compounds that regulate AR and Htt exon 1 aggregation, but only 2/10 of these compounds also inhibited polyglutamine peptide aggregation. This was unlikely to be due to increased aggregation kinetics of the polyglutamine peptide, because it produced FRET signal and visible inclusions that were intermediate between the peptides derived from AR (the least aggregation-prone) and Htt exon 1 (the most aggregation prone). In this case, common cellular mechanisms appear to influence AR and Htt exon 1 aggregation, as distinct from polyglutamine peptides fused to CFP/YFP.

Aggregation as a therapeutic target

Our results suggest that polyglutamine protein aggregation is an important aspect of cellular pathology, either as a primary event, or as a robust indicator of abnormal protein conformation. In prior work, we observed that Y-27632, an inhibitor of the rho-associated kinase p160ROCK, reduced AR and Htt exon 1 aggregation in this cell-based assay and also in the Drosophila Htt exon 1 model (35). Taken together...
with the results here, we have observed an overall assay confirmation rate in vivo of 6/11 compounds (∼54%). This suggests that the protein misfolding/aggregation phenomena measured in HEK293 cells reflect mechanisms similar to those that likely underlie Htt exon 1 neuropathology in Drosophila. This Drosophila system has predicted compounds (butyrate, SAHA) that function in mammals (45,46), and has also demonstrated the efficacy of other compounds that reduce toxicity in mice (Congo red, cystamine) (51–53). Thus, the validation of the FRET-based aggregation assay in this model suggests that in the future it may be useful for identifying and characterizing other effective compounds that reduce intracellular polyglutamine protein aggregation. Further analyses of leads identified in the FRET assay using full-length Drosophila and mouse models of polyglutamine disease will test more conclusively the utility of targeting aggregation in primary HTS.

FDA-approved compounds identified

This screen has identified three FDA-approved compounds that are effective as aggregation inhibitors in vitro, and in a Drosophila model in vivo: fosfosal, levonordenfin and nadolol. These compounds may thus be of particular interest for further study. Although it appears unlikely that the side effects of these drugs could be tolerated on a chronic basis, they may represent important leads for chemical optimization and characterization of mechanisms of action. Gefitinib is among the several putative inhibitors of EGFR signaling, and has been recently approved for treatment of non-small cell lung cancer (54). Another EGFR inhibitor (compound #3) functioned in Drosophila, although gefitinib did not. Although the activity of four putative EGFR antagonists in our system suggests a common mechanism of action, it will take further study to confirm their molecular basis. Clearly, it will be informative to test the orally available compounds (fosfosal, gefitinib, nadolol) in other Drosophila and mouse models, as they may represent important leads that could be rapidly translated into the clinic.

CONCLUSION

Despite more than 14 years of studying polyglutamine diseases, the development of novel therapies has been frustratingly slow. In part, this reflects a paucity of clearly defined molecular mechanisms of pathophysiology. However, the aggregation of polyglutamine proteins has emerged as an important molecular indicator of pathology, and may serve as an important therapeutic target. Thus, this work may advance drug discovery and our understanding of pathogenesis by providing an effective cellular assay of molecular pathology, with predictive value in vivo, that will help guide the identification, characterization and refinement of novel therapies. In addition, the specific compounds identified, and the cellular mechanisms they implicate, may provide exciting new pathways for genetic and pharmacologic approaches to identify and study inhibitors of polyglutamine pathogenesis.

MATERIALS AND METHODS

Plasmid construction

The plasmids AR(Q25)CFP/YFP, AR(Q65)CFP/YFP, Htt exon 1(25)CFP/YFP, Htt exon 1(72)CFP/YFP were previously described (35). Pure polyglutamine expression constructs containing 19 or 56 glutamines fused to CFP and YFP were a kind gift from Yoshitaka Nagai, M.D., Ph.D.

Detergent fractionation and size exclusion chromatography

HEK293 cells were cultured in a six-well plate (Costar) and transfected with 1.2 μg of total DNA consisting of 0.3 μg of p6RARN127CFP and 0.9 μg of p6RARN127YFP. Cells were cultured for 48 h. Cells were harvested by washing the plate in PBS/5 mM EDTA. At this time, ice-cold lysis buffer was prepared consisting of PBS with 1% Triton X-100 (Sigma) and protease inhibitor cocktail. Pellets were resuspended in 400 μl of lysis buffer, and lysis performed by 3 × 10 s sonication. Total protein from the lysate was determined in each sample according to the Bradford method, and samples were diluted with lysis buffer to obtain equivalent amounts of total protein. The samples were centrifuged at 1000 or 14 000 g for 5 min at 4°C. A total of 150 μl of the supernatant was removed for CFP and FRET measurements. Signal was measured from 70 μl of the lysate using a SAFIRE Fluorescence Plate Reader (Tecan Inc.) according to methods described previously (35).

For size exclusion chromatography, the Superdex 200 HR 10/30 size exclusion column (Amersham Biosciences) has an optimal separation range of 10 000–600 000 molecular weight and an exclusion limit of 1.3 × 106. The void volume of the column was first determined using Blue Dextran 2000 (Amersham Biosciences). It was then standardized using the manufacturer’s protocol. For each run, the column was equilibrated with 35 ml of PBS. For the experiment, two wells of a six-well dish were transfected with 1.2 μg of p6RARN127(25)YFP and 1.2 μg of p6RARN127(65)YFP. Cells were cultured for 48 h and harvested as described before. Lysates were prepared as before, and the samples were centrifuged at 14 000g. The size exclusion column was loaded with 500 μl of the supernatant for each protein, and the column was run using an FPLC at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and fluorescence measurements were performed immediately on the plate reader.

Screening of small molecule libraries

The ACL comprises biologically active small molecules selected for their lack of cellular toxicity. The library of kinase inhibitors was provided by Kevan Shokat, Ph.D. (UCSF) and the NINDS Custom Collection was obtained from Microsource. Daughter plates were created in duplicate from the 384-well parent microtiter plates that contained the compounds at concentrations of 10 or 5 mM, using robotic liquid handling system available through the core facility at UCSF. The compounds were aliquoted in black, clear-bottom 96-well plates (Costar™ 3603) at a concentration of...
10 μM. To screen the library, HEK293 were plated in 10 cm plates to obtain ~80% confluency on the day of transfection. Each plate was transfected with 0.7 μg of p6RAR127(65)CFP and 2.1 μg p6RAR127(65)YFP, and 0.7 μg p6RGRΔ (13) using 50 μl Plus reagent and 67 μl Lipofectamine according to manufacturer’s protocol (Invitrogen). After 3 h, complete medium was added and the plates were incubated overnight. The next day, cells were counted and plated in the duplicate 96-well daughter plates containing single aliquots of compounds from the libraries of daughter plates at a density of 140 000 cells/well (200 μl), after adding 100 nM dexamethasone. Each plate contained wells treated with Y-27632 (50 μM) as a positive control, and cells transfected only with unexpanded p6RAR(Q25)CFP/YFP constructs. This served as a reference point for background FRET levels without aggregation (a theoretical maximum effect on aggregation inhibition). The cells were then cultured for ~24 h to allow for aggregation and nuclear inclusion formation. After this time the cells were fixed using 75% 4% paraformaldehyde and washed with 150 μl PBS. CFP, YFP and FRET measurements were then performed. Mock-transfected cells were used to control for autofluorescence and light scattering. To control for donor values were determined for each sample (SMPL) were first subtracted from the raw data. Corrected FRET/donor values were determined for each sample (SMPL) according to the formula:

\[
\text{FRET/donor} = \frac{(\text{SMPL}_{435} - X \times (\text{SMPL}_{485} - \text{SMPL}_{435}))}{\text{SMPL}_{485} - \text{SMPL}_{435}}
\]

where \(X = \frac{\text{YFP}_{435} \times \text{YFP}_{485}}{\text{YFP}_{485} \times \text{YFP}_{435}}\).

Screening details from each plate are provided in Supplementary Material, Table S2.

Htt exon 1 detergent fractionation from inducible PC-12 cells

An inducible model of Htt exon 1 expression in PC-12 cells has been previously described (51). PC-12 cells were cultured in six-well plates in the presence of test compounds or vehicle control (DMSO) for 48 h. At this time cells were harvested by washing the plates with 5 mM EDTA/PBS. Cells were recovered by centrifugation at 1000 g in a desktop centrifuge. Cell lysates were prepared by syringe lysis of cells in buffer containing PBS, 1% Triton X-100 and protease inhibitor cocktail. A sample of lysate was removed for protein quantification by Bradford analysis. The remaining lysate was subjected to centrifugation at 14 000 g for 10 min at 4°C. The top 80% of the supernatant was removed, and the remaining supernatant fraction. The remaining supernatant was discarded, and the pellet was washed twice in lysis buffer. All remaining buffer was removed, and the pellet was resuspended in SDS-sample buffer and boiled for 5 min. Equivalent amounts of the supernatant and pellet fractions, normalized to total protein, were resolved by SDS–PAGE and western blot with anti-GFP antibody (Santa Cruz). The gels were imaged using chemiluminescent substrate (Pierce) in conjunction with a digital imager. This experiment was repeated three times. Band intensities were determined based on chemiluminescent signal. For each experiment, the pellet band intensity was compared with the corresponding supernatant signal intensity across all compounds. The pellet/supernatant ratio for cells induced with ponasterone, but treated only with vehicle was determined and set arbitrarily to 1. All other ratios were thus normalized to this value, which allowed cross-comparison between the various experiments.

Assay of neurodegeneration in Drosophila

Transgenic flies were used that harbor a human huntingtin exon 1 fragment with 93 glutamines [htt exon 1 (Q93)] that is expressed in all fly neurons under the control of the elav:Gal4 driver. Flies were treated by maintaining adults on food containing compounds as indicated, or no drug supplement. Flies expressing the transgene display progressive degeneration of the photoreceptor neurons, which is revealed by a time-dependent decrease in the number of rhabdomeres per ommatidium (26). Normal fly eyes have seven visible rhabdomeres, whereas flies expressing htt exon 1 (Q93) display reduced number of rhabdomeres. The number of rhabdomeres in treated versus untreated flies was analyzed 7 days post-eclosion by the pseudopupil technique as described (26,47). Analyses were performed blinded to the compounds being tested. Approximately 50–60 ommatidia were analyzed for rhabdomere content on each of 10–12 flies from each treatment group, and the average number of rhabdomeres per ommatidium in each individual was determined. Percent surviving rhabdomeres were calculated as 100 × (E7 – C7)/ (C0 − C7) with the number of rhabdomeres in the experimental (E) vs. control (C) groups at 0 or 7 days.

Purchased compounds

All the purchased compounds were obtained from Sigma.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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6. Authors state that they have increased the number of references.


