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 $\sim 80–100$Å. 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
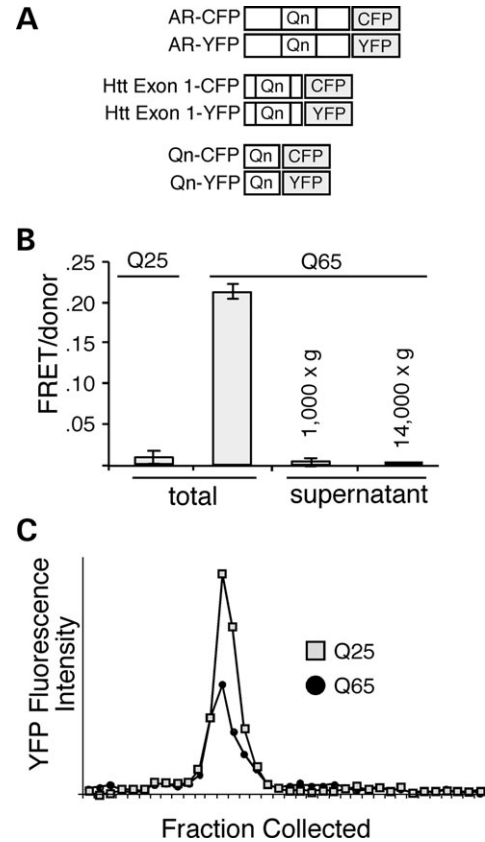

Figure 1. FRET assay detects detergent-soluble and detergent-insoluble polyglutamine aggregation. (**A**) Diagrams of AR constructs: truncated AR contains the first 127 amino acids of AR (AR(Qn)) with either an unexpanded ($n = 25$) or expanded ($n = 65$) tract of glutamines. Htt exon 1 consists of the first exon of the *Htt* gene with unexpanded ($n = 24$) or expanded ($n = 72$) glutamines. The pure polyglutamine peptide consists of unexpanded ($n = 24$) or expanded ($n = 53$) tract of glutamines. The truncated peptides were fused to coding sequences of CFP and YFP, respectively. (**B**) HEK293 cells were transfected with expanded or unexpanded AR(Qn)CFP/YFP, and a lysate was made using 1% Triton. Detergent fractionation demonstrated that no significant FRET signal derived from AR(Q25)CFP/YFP, whereas AR(Q65)CFP/YFP had strong signal present in the total lysate. After centrifugation, the FRET signal from Q65 disappeared from the supernatant, indicating that most of it derived from detergent-insoluble complexes (large inclusions). Error bars represent the range from two different samples. (**C**) Supernatant of lysates as in (B), with expanded or unexpanded AR(Qn)YFP, were passed over a Superdex 200 size exclusion column. Fractions were collected and analyzed by measuring YFP. Both expanded and unexpanded proteins eluted from the column as monomers with an appropriate apparent molecular weight of $\sim 70–90$ kDa. There was no evidence of multimers or large MW structures in the Q65 supernatant fraction. Reduced signal in the Q65 fraction is due to prior pelleting of aggregated material.

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 000g 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).

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 14 000*g* 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 a final concentration of 10 μM, at which most are active, but are not toxic to the cells.

We have previously determined that co-expression of a mutant form of the glucocorticoid receptor (GRΔ) with polyglutamine proteins induces nuclear inclusion formation in cultured cells upon dexamethasone stimulation (13). HEK293 cells were co-transfected with AR(Q65)CFP/YFP and GRΔ to facilitate nuclear inclusion formation (13,35). Internal controls on each plate included wells treated with Y-27632 as a positive control, untreated wells for comparison, and cells transfected only with unexpanded AR(Q25)CFP/YFP constructs as a reference point for no aggregation. FRET measurements for each plate were normalized between 100% (FRET levels from AR(Q65)CFP/YFP with no drug treatment) and 0% (FRET levels from AR(Q25)CFP/YFP), allowing for comparisons between all plates, and accounting for experimental variation over time. Figure 2 shows a flow chart of the screening steps. Only compounds that showed a decrease of at least 15% in each of the duplicates, with an average reduction of at least 20% were considered for further analysis. We readily identified toxic compounds during the screen on the basis of loss of absolute CFP and YFP signal; these were discarded. The average reduction in aggregation for all compounds was ~6.75%, with an SD of 6%. Thus compounds selected in the primary screen were at least 2 SD from the mean. Hits from the initial screens were tested again at two concentrations, 10 and 3 μM, in four replicates. This eliminated ~80% of the initial hits. Effective compounds from the second round were then obtained from commercial sources to perform detailed dose-response studies, and to determine the half-maximal effective concentrations (EC_{50}). This eliminated ~50% of the remaining hits, and consequently we identified 10 compounds that

Annotated Compound Library
Kinase Inhibitor Library
NINDS Compound Collection
Total=4140 Compounds

↓

Primary Screen at 10μM
in Duplicate

↓

116 hits

↓

Retest Hits at 10μM and 3μM
Quadruplicates for Each Condition

↓

21 hits

↓

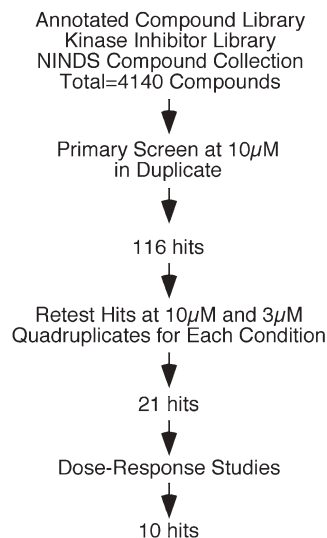Dose-Response Studies

↓

10 hits

Figure 2. Flow chart describing the screening steps. For the primary screen 10 μM of each compound was tested in duplicate on separate plates, and for the second round of screening two doses were tested 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.

consistently reduced AR(Q65)CFP/YFP aggregation (Table 1). In each case, our EC_{50} 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 $P \leq 0.005$.

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

Table 1. List of hits from each library

Library source	Compound	Mechanism	Reduction AR aggregation (%)	Reduction htt aggregation (%)	Reduction PolyQ peptide aggregation (%)	EC_{50} for AR
ACL	Ac-YVAD-cmk (interleukin lb-converting enzyme II inhibitor)	Caspase 1 inhibitor	29	32	15	150 nM
Kinase Inhibitor Library	Piceatannol	Syk protein tyrosine kinase inhibitor	23	28	No significant effect	100 nM
	Todralazine	Uncertain	19	15	No significant effect	5 μM
	EGFR inhibitor no. 3	EGFR tyrosine kinase inhibitor	30	21	No significant effect	20 μM
	EGFR inhibitor no. 4	EGFR tyrosine kinase inhibitor	28	15	No significant effect	2.5 μM
	Gefitinib	EGFR tyrosine kinase inhibitor	27	20	No significant effect	80 nM
NINDS	Molsidomine	Nitric oxide donor	20	15	No significant effect	6 nM
	Fosfosal	Uncertain	21	20	15	2 μM
	Nadolol	Beta-adrenergic blocker	19	18	No significant effect	20 nM
	Levonordefrin	Alpha-adrenergic agonist	21	18	No significant effect	80 nM

HEK293 cells were co-transfected in 10 cm plates with AR(Q65)CFP/YFP, and a mutant form of the glucocorticoid receptor (GRΔ) 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 exon1, whereas only two of the hits inhibited aggregation of polyglutamine peptides. EC_{50} values were determined with detailed dose-response studies for AR(Q65)CFP/YFP, with a maximum $P \leq 0.004$ (see Fig. 4).

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 $β_1$ antagonist (atenolol), and a more selective $β_2$ antagonist (butoxamine). However, neither reduced aggregation, suggesting that the effects of nadolol might be off-target, or that inhibition requires simultaneous $β_1$ and $β_2$ 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
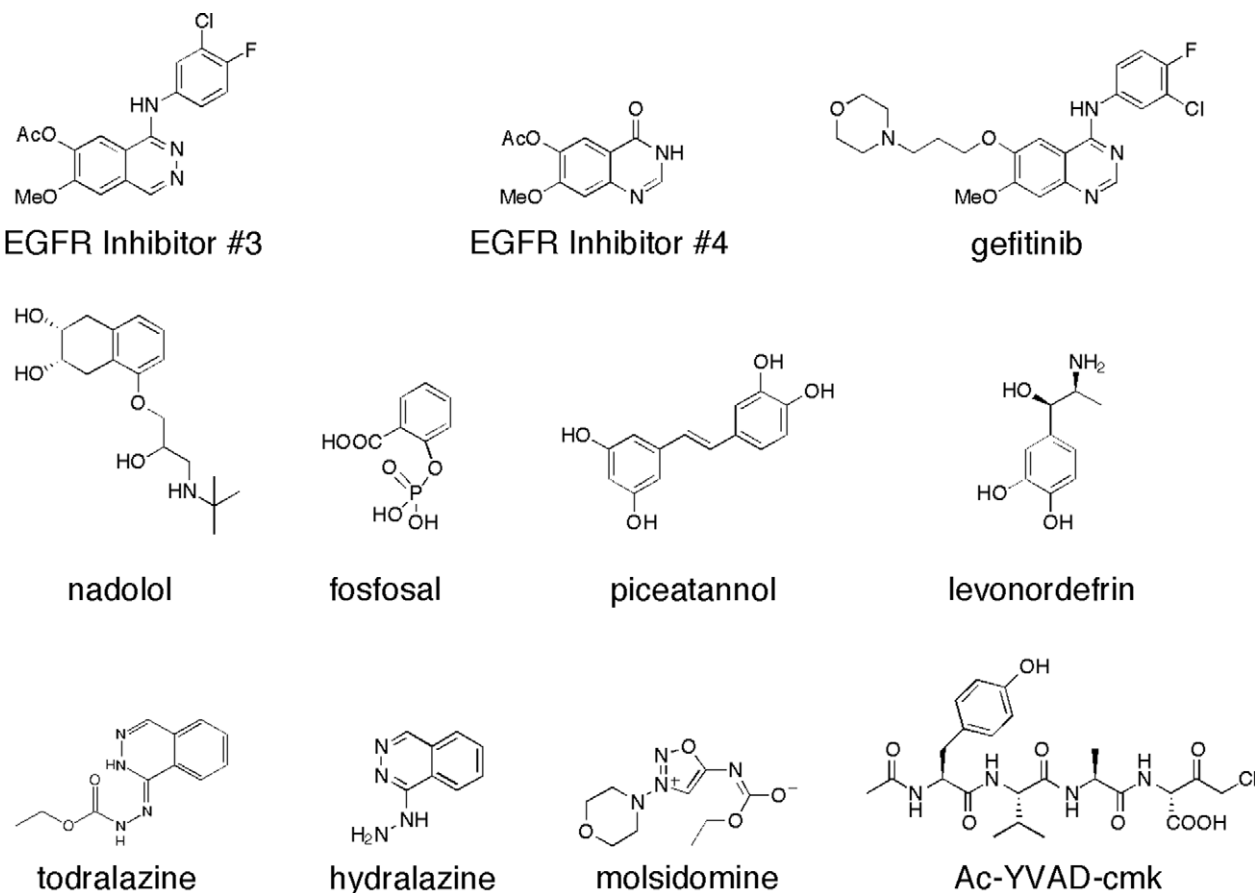
EGFR Inhibitor #3   EGFR Inhibitor #4   gefitinib

nadolol   fosfosal   piceatannol   levonordefrin

todralazine   hydralazine   molsidomine   Ac-YVAD-cmk

Figure 3. Structures of validated hits from the screen. An additional compound, hydralazine, is pictured because this compound was tested in *Drosophila*.

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-
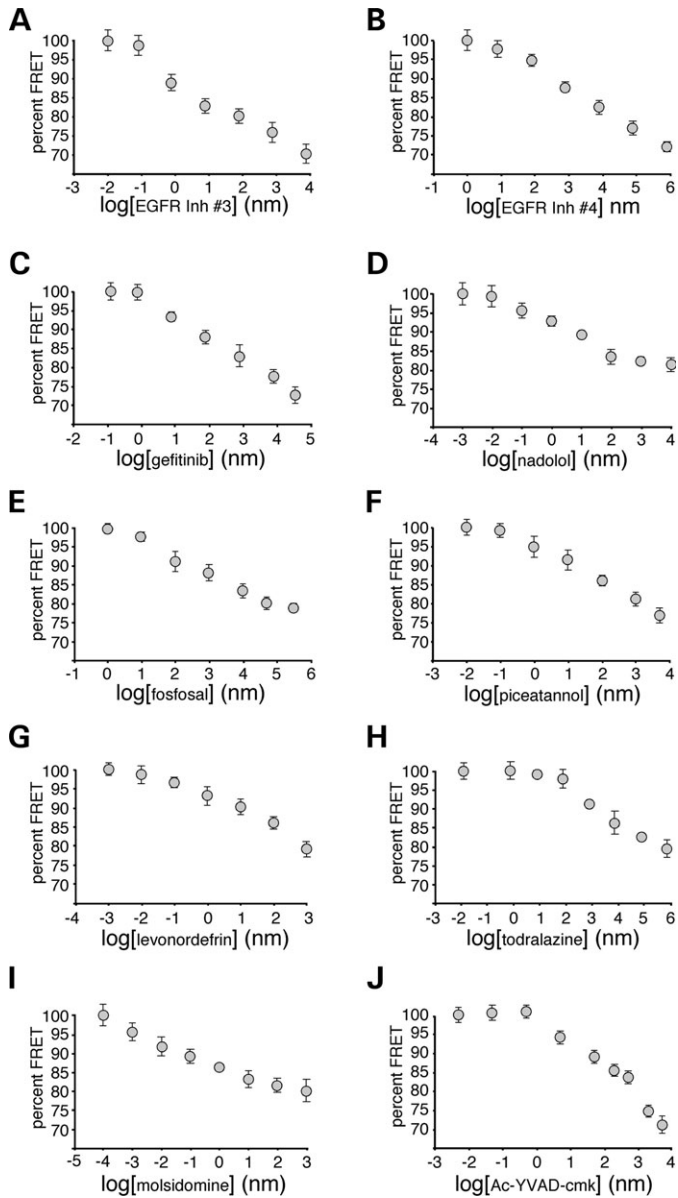
Figure 4. Dose-response of hits from the primary screen. HEK293 cells were transfected with AR(65)CFP/YFP and cultured in the presence of test compounds for 24 h at the indicated concentrations. FRET values were determined on the fluorescence plate reader. No compound showed >30% reduction in aggregation. Compounds tested were as follows: (**A**) EGFR inhibitor no. 3; (**B**) EGFR inhibitor no. 4; (**C**) gefitinib; (**D**) nadolol; (**E**) fosfosal; (**F**) piceatannol; (**G**) levonordefrin; (**H**) todralazine; (**I**) molsidomine; (**J**) ac-YVAD-cmk. Error bars represent the SD for each point (note: for some points the circle obscures the error bars).

Figure 5. Test of compounds in a PC-12 model of Htt exon 1 aggregation. PC-12 cells expressing Htt(Q92) exon 1-GFP from an ECR-inducible promoter were cultured with 1 μM ponasterone for 48 h in the presence or absence of test compounds at 10 μM. Y-27632 was included as a positive control at 50 μM. Cells were then subjected to detergent fractionation and equivalent amounts of the supernatant and pellet fractions were resolved by SDS–PAGE and western blot. Gels were developed using chemiluminescent substrate, and the bands were directly imaged using a CCD camera. The ratio of pellet to supernatant was determined for each compound. Within an experiment, all compounds were compared with the pellet:supernatant ratio for untreated cells (defined as unity), which was used to normalize separate experiments. The fractionation was repeated three times, and the SD of each ratio was determined. Only todralazine failed to reduce detergent insolubility.

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 mo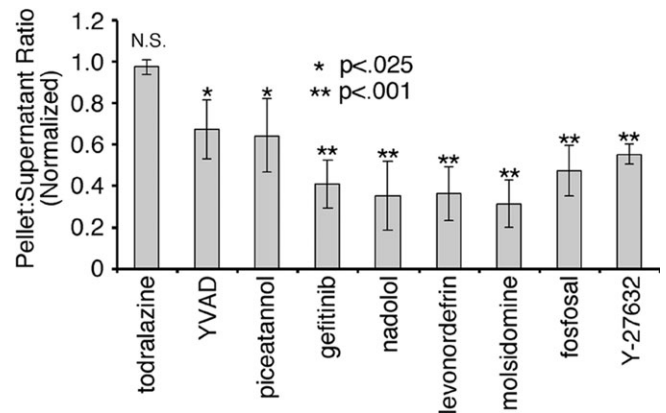lecules: the ACL, a collection of kinase inhibitors and the NINDS Compound Collection. Compounds were screened in duplicate for suppression of AR aggregation. Hits were validated by determining dose responses, and, where possible, by alternative pharmacologic inhibition. Hits from this primary assay 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.
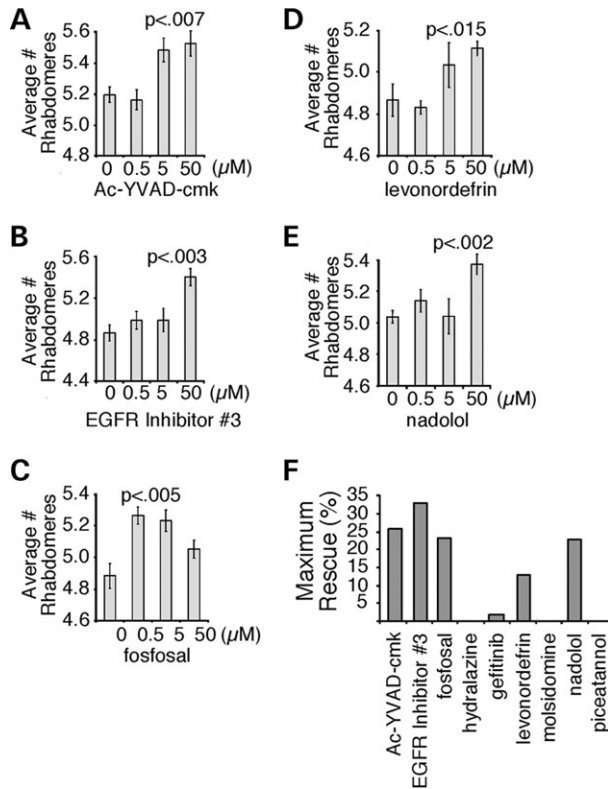
Figure 6. Analysis of hits in a *Drosophila* model of neurodegeneration. Hits derived from the cellular assay were tested in the *Drosophila* model. Flies harboring a UAS:Htt exon 1 (Q93) gene were crossed to those harboring elav:Gal4, which results in pan-neuronal expression of Htt exon 1. This produces 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 μM). (**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.

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 overtly 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.

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, levonordefrin 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 000g 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 1.3×10^6. 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 p6RARN127(65)CFP and 2.1 µg p6RARN127(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 µl 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 bleedthrough and crossover excitation, CFP and YFP controls were transfected in a 12-well plate with 0.3 µg p6RARN127(25)CFP and 0.3 µg p6RARN127(25)YFP, respectively. The background CFP, YFP and FRET signals were first subtracted from the raw data. Corrected FRET/donor values were determined for each sample (SMPL) according to the formula:

$$FRET/donor = \{SMPL_{435/527} \\ - X * (SMPL_{485/527})\}/SMPL_{435/485}$$

where $X = YFP_{435/527}/YFP_{485/527}$.

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 $1000g$ 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 000g for 10 min at 4°C. The top 80% of the supernatant was removed, and constituted the 'supernatant' fraction. The remaining supernatant was discarded, and the pellet was washed two times 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 $100 \times (E_7 - C_7)/(C_0 - C_7)$ 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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Conflict of Interest statement. The authors state that they have no conflicts of interest with this work.

REFERENCES

1. Harding, A.E., Thomas, P.K., Baraitser, M., Bradbury, P.G., Morgan-Hughes, J.A. and Ponsford, J.R. (1982) X-linked recessive bulbospinal neuropathy: a report of ten cases. *J. Neurol. Neurosrurg. Psychiat.*, **45**, 1012–1019.

2. Kennedy, W.R., Alter, M. and Sung, J.H. (1968) Progressive proximal spinal and bulbar muscular atrophy of late onset. A sex-linked recessive trait. *Neurology*, **18**, 671–680.

3. La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E. and Fischbeck, K.H. (1991) Androgen receptor gene mutations in X-linked spinobulbar muscular atrophy. *Nature*, **352**, 77–79.

4. Group, H.s.D.C.R. (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell*, **72**, 971–983.

5. Cummings, C.J., Reinstein, E., Sun, Y., Antalffy, B., Jiang, Y., Ciechanover, A., Orr, H.T., Beaudet, A.L. and Zoghbi, H.Y. (1999) Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron*, **24**, 879–892.

6. Zoghbi, H.Y. and Orr, H.T. (1999) Polyglutamine diseases: protein cleavage and aggregation. *Curr. Opin. Neurobiol.*, **9**, 566–570.

7. Cummings, C.J., Sun, Y., Opal, P., Antalffy, B., Mestril, R., Orr, H.T., Dillmann, W.H. and Zoghbi, H.Y. (2001) Over-expression of inducible HSP70 chaperone suppresses neuropathology and improves motor function in SCA1 mice. *Hum. Mol. Genet.*, **10**, 1511–1518.

8. Arrasate, M., Mitra, S., Schweitzer, E.S., Segal, M.R. and Finkbeiner, S. (2004) Inclusion body formation reduces levels of mutant huntingtin and the risk of neuronal death. *Nature*, **431**, 747–748.

9. Klement, I.A., Skinner, P., Kaytor, M.D., Yi, H., Hersch, S.M., Clark, H.B., Zoghbi, H.Y. and Orr, H.T. (1998) Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. *Cell*, **95**, 41–53.

10. Cowan, K.J., Diamond, M.I. and Welch, W.J. (2003) Polyglutamine protein aggregation and toxicity are linked to the cellular stress response. *Hum. Mol. Genet.*, **12**, 1377–1391.

11. Welch, W.J. and Diamond, M.I. (2001) Glucocorticoid modulation of androgen receptor nuclear aggregation and cellular toxicity is associated with distinct forms of soluble expanded polyglutamine protein. *Hum. Mol. Genet.*, **10**, 3063–3074.

12. Bence, N.F., Sampat, R.M. and Kopito, R.R. (2001) Impairment of the ubiquitin-proteasome system by protein aggregation. *Science*, **292**, 1552–1555.

13. Diamond, M.I., Robinson, M.R. and Yamamoto, K.R. (2000) Regulation of expanded polyglutamine protein aggregation and nuclear localization in glucocorticoid receptor. *Proc. Natl Acad. Sci.*, **97**, 657–661.

14. Wyttenbach, A., Carmichael, J., Swartz, J., Furlong, R.A., Narain, Y., Rankin, J. and Rubinsztein, D.C. (2000) Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. *Proc. Natl Acad. Sci.*, **97**, 2898–2903.

15. Meriin, A.B., Mabuchi, K., Gabai, V.L., Yaglom, J.A., Kazantsev, A. and Sherman, M.Y. (2001) Intracellular aggregation of polypeptides with expanded polyglutamine domain is stimulated by stress-activated kinase MEKK1. *J. Cell Biol.*, **153**, 851–864.

16. Chen, H.K., Fernandez-Funez, P., Acevedo, S.F., Lam, Y.C., Kaytor, M.D., Fernandez, M.H., Aitken, A., Skoulakis, E.M., Orr, H.T., Botas, J. and Zoghbi, H.Y. (2003) Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. *Cell*, **113**, 457–468.

17. Humbert, S., Bryson, E.A., Cordelieres, F.P., Connors, N.C., Datta, S.R., Finkbeiner, S., Greenberg, M.E. and Saudou, F. (2002) The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. *Cell*, **2**, 831–837.

18. Sakahira, H., Breuer, P., Hayer-Hartl, M.K. and Hartl, F.U. (2002) Molecular chaperones as modulators of polyglutamine protein aggregation and toxicity. *Proc. Natl Acad. Sci. USA*, **99**, 16412–16418.

19. Wang, J., Gines, S., MacDonald, M.E. and Gusella, J.F. (2005) Reversal of a full-length mutant huntingtin neuronal cell phenotype by chemical inhibitors of polyglutamine-mediated aggregation. *BMC Neurosci.*, **6**, 1.

20. Georgalis, Y., Starikov, E.B., Hollenbach, B., Lurz, R., Scherzinger, E., Saenger, W., Lehrach, H. and Wanker, E.E. (1998) Huntingtin aggregation monitored by dynamic light scattering. *Proc. Natl Acad. Sci. USA*, **95**, 6118–6121.

21. Piccioni, F., Roman, B.R., Fischbeck, K.H. and Taylor, J.P. (2004) A screen for drugs that protect against the cytotoxicity of polyglutamine-expanded androgen receptor. *Hum. Mol. Genet.*, **13**, 437–446.

22. Zhang, X., Smith, D.L., Meriin, A.B., Engemann, S., Russel, D.E., Roark, M., Washington, S.L., Maxwell, M.M., Marsh, J.L., Thompson, L.M. *et al.* (2005) A potent small molecule inhibits polyglutamine aggregation in Huntington's disease neurons and suppresses neurodegeneration *in vivo*. *Proc. Natl Acad. Sci. USA*, **102**, 892–897.

23. Aiken, C.T., Tobin, A.J. and Schweitzer, E.S. (2004) A cell-based screen for drugs to treat Huntington's disease. *Neurobiol. Dis.*, **16**, 546–555.

24. Heiser, V., Engemann, S., Brocker, W., Dunkel, I., Boeddrich, A., Waelter, S., Nordhoff, E., Lurz, R., Schugardt, N., Rautenberg, S. *et al.* (2002) Identification of benzothiazoles as potential polyglutamine aggregation inhibitors of Huntington's disease by using an automated filter retardation assay. *Proc. Natl Acad. Sci.*, **99**, 16400–16406.

25. Kaytor, M.D., Byam, C.E., Tousey, S.K., Stevens, S.D., Zoghbi, H.Y. and Orr, H.T. (2005) A cell-based screen for modulators of ataxin-1 phosphorylation. *Hum. Mol. Genet.*, **14**, 1095–1105.

26. Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M. *et al.* (2001) Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in *Drosophila*. *Nature*, **413**, 739–743.

27. Smith, D.L., Portier, R., Woodman, B., Hockly, E., Mahal, A., Klunk, W.E., Li, X.J., Wanker, E., Murray, K.D. and Bates, G.P. (2001) Inhibition of polyglutamine aggregation in R6/2 HD brain slices-complex dose-response profiles. *Neurobiol. Dis.*, **8**, 1017–1026.

28. Heiser, V., Engemann, S., Boeddrich, A., Nordhoff, E., Lurz, R., Schugardt, N., Lehrach, H. and Wanker, E.E. (2000) Inhibition of huntingtin fibrillogenesis by specific antibodies and small molecules: implications for Huntington's disease therapy. *Proc. Natl Acad. Sci. USA*, **97**, 6739–6744.

29. Kaemmerer, W.F., Rodrigues, C.M., Steer, C.J. and Low, W.C. (2001) Creatine-supplemented diet extends Purkinje cell survival in spinocerebellar ataxia type 1 transgenic mice but does not prevent the ataxic phenotype. *Neuroscience*, **103**, 713–724.

30. Wellington, C.L., Singaraja, R., Ellerby, L., Savill, J., Roy, S., Leavitt, B., Cattaneo, E., Hackam, A., Sharp, A., Thornberry, N. *et al.* (2000) Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and non-neuronal cells. *J. Biol. Chem.*, **275**, 19831–19838.

31. Kim, S.J., Kim, T., Hong, S., Rhim, H., Kim, I.Y. and Kang, S. (2003) Oxidative stimuli affect polyglutamine aggregation and cell death in human mutant ataxin-1-expressing cells. *Neurosci. Lett.*, **348**, 21–24.

32. Zeron, M.M., Fernandes, H.B., Krebs, C., Shehadeh, J., Wellington, C.L., Leavitt, B.R., Baimbridge, K.G., Hayden, M.R. and Raymond, L.A. (2004) Potentiation of NMDA receptor-mediated excitotoxicity linked with intrinsic apoptotic pathway in YAC transgenic mouse model of Huntington's disease. *Mol. Cell. Neurosci.*, **25**, 469–479.

33. Ona, V.O., Li, M., Vonsattel, J.P., Andrews, L.J., Khan, S.Q., Chung, W.M., Frey, A.S., Menon, A.S., Li, X.J., Stieg, P.E. *et al.* (1999) Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. *Nature*, **399**, 204–207.

34. Apostol, B.L., Kazantsev, A., Raffioni, S., Illes, K., Pallos, J., Bodai, L., Slepko, N., Bear, J.E., Gertler, F.B., Hersch, S. *et al.* (2003) A cell-based assay for aggregation inhibitors as therapeutics of polyglutamine-repeat disease and validation in *Drosophila*. *Proc. Natl Acad. Sci.*, **100**, 5950–5955.

35. Pollitt, S.K., Pallos, J., Shao, J., Desai, U.A., Aye Ma, Aye, Thompson, L.M., Marsh, J.L. and Diamond, M.I. (2003) A rapid cellular FRET assay of polyglutamine aggregation identifies a novel inhibitor. *Neuron*, **40**, 685–694.

36. Kim, S., Nollen, E.A., Kitagawa, K., Bindokas, V.P. and Morimoto, R.I. (2002) Polyglutamine protein aggregates are dynamic. *Nat. Cell Biol.*, **4**, 826–831.

37. Jorissen, R.N., Walker, F., Pouliot, N., Garrett, T.P., Ward, C.W. and Burgess, A.W. (2003) Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp. Cell. Res.*, **284**, 31–53.

38. Yano, S., Kondo, K., Yamaguchi, M., Richmond, G., Hutchison, M., Wakeling, A., Averbuch, S. and Wadsworth, P. (2003) Distribution and

function of EGFR in human tissue and the effect of EGFR tyrosine kinase inhibition. *Anticancer Res.*, **23**, 3639–3650.

39. Nitz, R.E. and Fiedler, V.B., (1987) Molsidomine: alternative approaches to treat myocardial ischemia. *Pharmacotherapy*, **7**, 28–37.

40. Greenberg, S., (1999) Modular components of phagocytosis. *J. Leukoc. Biol.*, **66**, 712–717.

41. Jiang, K., Zhong, B., Ritchey, C., Gilvary, D.L., Hong-Geller, E., Wei, S. and Djeu, J.Y. (2003) Regulation of Akt-dependent cell survival by Syk and Rac. *Blood*, **101**, 236–244.

42. Narula, S.S., Yuan, R.W., Adams, S.E., Green, O.M., Green, J., Philips, T.B., Zydowsky, L.D., Botfield, M.C., Hatada, M., Laird, E.R. *et al*. (1995) Solution structure of the C-terminal SH2 domain of the human tyrosine kinase Syk complexed with a phosphotyrosine pentapeptide. *Structure*, **3**, 1061–1073.

43. Yamada, T., Fujieda, S., Yanagi, S., Yamamura, H., Inatome, R., Sunaga, H. and Saito, H. (2001) Protein-tyrosine kinase Syk expressed in human nasal fibroblasts and its effect on RANTES production. *J. Immunol.*, **166**, 538–543.

44. Minamiyama, M., Katsuno, M., Adachi, H., Waza, M., Sang, C., Kobayashi, Y., Tanaka, F., Doyu, M., Inukai, A. and Sobue, G. (2004) Sodium butyrate ameliorates phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. *Hum. Mol. Genet.*, **13**, 1183–1192.

45. Hockly, E., Richon, V.M., Woodman, B., Smith, D.L., Zhou, X., Rosa, E., Sathasivam, K., Ghazi-Noori, S., Mahal, A., Lowden, P.A. *et al*. (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. *Proc. Natl Acad. Sci. USA*, **100**, 2041–2046.

46. Ferrante, R.J., Kubilus, J.K., Lee, J., Ryu, H., Beesen, A., Zucker, B., Smith, K., Kowall, N.W., Ratan, R.R., Luthi-Carter, R. *et al*. (2003) Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. *J. Neurosci.*, **23**, 9418–9427.

47. Franceschini, N. and Kirschfeld, K. (1971) Pseudopupil phenomena in the compound eye of *Drosophila*. *Kybernetik*, **9**, 159–182.

48. Chen, S., Berthelier, V., Yang, W. and Wetzel, R. (2001) Polyglutamine aggregation behavior *in vitro* supports a recruitment mechanism of cytotoxicity. *J. Mol. Biol.*, **311**, 173–182.

49. Thakur, A.K., Yang, W. and Wetzel, R. (2004) Inhibition of polyglutamine aggregate cytotoxicity by a structure-based elongation inhibitor. *FASEB J.*, **18**, 923–925.

50. Yang, W., Dunlap, J.R., Andrews, R.B. and Wetzel, R. (2002) Aggregated polyglutamine peptides delivered to nuclei are toxic to mammalian cells. *Hum. Mol. Genet.*, **11**, 2905–2917.

51. Apostol, B.L., Kazantsev, A., Raffioni, S., Illes, K., Pallos, J., Bodai, L., Slepko, N., Bear, J.E., Gertler, F.B., Hersch, S. *et al*. (2003) A cell-based assay for aggregation inhibitors as therapeutics of polyglutamine-repeat disease and validation in *Drosophila*. *Proc. Natl Acad. Sci. USA*, **100**, 5950–5955.

52. Karpuj, M.V., Becher, M.W., Springer, J.E., Chabas, D., Youssef, S., Pedotti, R., Mitchell, D. and Steinman, L. (2002) Prolonged survival and decreased abnormal movements in transgenic model of Huntington disease, with administration of the transglutaminase inhibitor cystamine. *Nat. Med.*, **8**, 143–149.

53. Sanchez, I., Mahlke, C. and Yuan, J. (2003) Pivotal role of oligomerization in expanded polyglutamine neurodegenerative disorders. *Nature*, **421**, 373–379.

54. Ciardiello, F., De Vita, F., Orditura, M. and Tortora, G. (2004) The role of EGFR inhibitors in non-small cell lung cancer. *Curr. Opin. Oncol.*, **16**, 130–135.