IDENTIFICATION OF ANTI-PRION COMPOUNDS AS EFFICIENT INHIBITORS OF POLYGLUTAMINE PROTEIN AGGREGATION IN A ZEBRAFISH MODEL

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Running Title: Inhibitors of polyglutamine protein aggregation in zebrafish

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Several neurodegenerative diseases, including Huntington’s disease (HD), are associated with aberrant folding and aggregation of polyglutamine (polyQ) expansion proteins. Here we established the zebrafish, *Danio rerio*, as a vertebrate HD model permitting the screening for chemical suppressors of polyQ aggregation and toxicity. Upon expression in zebrafish embryos, polyQ-expanded fragments of huntingtin (htt) accumulated in large SDS-insoluble inclusions, reproducing a key feature of HD pathology. Real-time monitoring of inclusion formation in the living zebrafish indicated that inclusions grow by rapid incorporation of soluble htt species. Expression of mutant htt increased the frequency of embryos with abnormal morphology and the occurrence of apoptosis. Strikingly, apoptotic cells were largely devoid of visible aggregates, suggesting that soluble oligomeric precursors may instead be responsible for toxicity. As in non-vertebrate polyQ disease models, the molecular chaperones, Hsp40 and Hsp70, suppressed both polyQ aggregation and toxicity. Using the newly established zebrafish model, two compounds of the *N*-benzylidene-benzohydrazide class directed against mammalian prion proved to be potent inhibitors of polyQ aggregation, consistent with a common structural mechanism of aggregation for prion and polyQ disease proteins.

**INTRODUCTION**

A common feature of neurodegenerative diseases, such as Parkinson’s disease, Alzheimer’s disease, and polyglutamine (polyQ) diseases, including Huntington’s disease (HD), is the accumulation and deposition of characteristic amyloid or amyloid-like fibrils of the respective disease proteins in the brain (1,2). In HD, it is the intracellular deposition of inclusion bodies (IBs) that is associated with the degeneration of neurons, primarily in the striatum region of the brain. These IBs contain fibrillar aggregates of the disease protein, huntingtin (htt), and likely represent the final manifestation of a multi-step aggregation pathway involving several intermediate species (3).

The pathogenic length of the polyQ stretch within the N-terminal segment of htt is ~38Q or greater, and correlates with its ability to form detergent insoluble fibrillar aggregates (4-6). N-terminal fragments containing the pathogenic polyQ stretch have been detected in inclusions in HD brain tissue, and their generation is considered to be a critical step in both fibril formation and toxicity (4,7). Molecular dynamics simulations of polyQ fragments suggest that the core structure of a htt fibril is composed of peptides arranged in a triangular β-helical structure (8). Such fibrillar structures would grow by attachment of further coils at the top and bottom ends of the β-helix.

Although IBs are closely associated with neurodegenerative disease, there has been considerable debate about the role of aggregates in pathogenesis. Increasing evidence suggests that IBs, and the fibrillar aggregates therein, may not represent the primary neurotoxic agent (1,9-11). Instead, soluble intermediates in the aggregation pathway are likely to be the key toxic species, giving rise to neurodegeneration through various mechanisms including inactivation of essential transcription factors and inhibition of the ubiquitin proteasome system (12,13). Much attention has therefore been focused on identifying means to disrupt or alter the polyQ aggregation pathway (14), and several compounds with anti-aggregation activity have been identified *in vitro* and in cell based assays (15,16).

We set out to develop a zebrafish HD model system suitable for whole organism validation of candidate compounds identified as aggregation inhibitors *in vitro*. The zebrafish has several key advantages over other *in vivo* models of HD. It is a vertebrate organism and contains homologues to many human genes, including the htt gene (17), making it a valuable tool to model human diseases such as HD. Additional important
advantages include: the ease and cost efficiency with which in vivo drug tests can be performed; the transparency of zebrafish embryos allowing for visualization of morphological and physiological features in the live embryo; and the aqueous environment of the zebrafish, which facilitates drug administration (18). Importantly, the zebrafish model system allows for the evaluation of drug effectiveness in a whole organism, taking into account the stability and cellular targeting of the tested compound, as well as the assessment of potential side effects of the drug at active concentrations.

Here, we describe the characterization of a newly established zebrafish model of HD. Mutant htt expressed in zebrafish embryos accumulated in large SDS-insoluble inclusions, recapitulating a cardinal feature of HD pathology. Mutant htt also increased the frequency of embryos with abnormal morphology and dead embryos, as well as the occurrence of apoptosis. As in non-vertebrate polyQ disease models, the molecular chaperones, Hsp40 and Hsp70, suppressed both polyQ aggregation and toxicity. Using this zebrafish model, two anti-prion compounds of the \( \text{N'} \)-benzylidene-benzohydrazide class were identified as novel inhibitors of polyQ aggregation, suggesting that polyQ aggregates and prions may share common structural epitopes. We also address, for the first time in a vertebrate model system, the controversial question of whether inclusion bodies of polyQ-expanded htt directly contribute to toxicity. Our observation that the majority of apoptotic cells were devoid of visible inclusions suggests that polyQ toxicity is not primarily caused by insoluble aggregates, but is more likely caused by soluble aggregation intermediates.

**EXPERIMENTAL PROCEDURES**

*Animal husbandry* - All experiments were performed in compliance with the guidelines of the German Council on Animal Care. Zebrafish were maintained, mated, and raised as described (19). Embryos were kept at 28°C and staged as described (20). The wild type line AB was used for all experiments.

Constructs and embryo injection - pCS2+Q25GFP and pCS2+Q102GFP were generated by subcloning the inserts from pYES2-htt(25 or 102Q)-GFP (21) into the AflIII and XbaI sites of the pCS2+-AflII vector. PCR mutagenesis of pCS2+Q25GFP was used to generate a Q4GFP fragment, which was cloned into NdeI and BstAPI cut pCS2+Q25GFP backbone. To generate pCS2+Hsp70-YFP, an Hsp70-YFP fragment derived from pEYFP-N1-Hsp70 (22) was cloned into EcoRI, XbaI cut pCS2+. The construct used for transcription of human Hdj1 (Hsp40) was a kind gift from A. Haacke; the \( \beta \)-Galactoside construct was purchased from Invitrogen. *In vitro* transcription of all these constructs was performed with the MessageMachine Kit (Ambion, TX), and the resulting mRNA injected at a concentration of 1 \( \mu \text{g/µl} \) into the yolk of embryos at the 1–2 cell stage. Co-injections were performed with 1 \( \mu \text{g/µl} \) polyQ-GFP mRNA and either 1 \( \mu \text{g/µl} \) \( \beta \)-Gal mRNA as a control or 0.5 \( \mu \text{g/µl} \) Hdj1 and 0.5 \( \mu \text{g/µl} \) Hsp70-YFP mRNA. Embryos were cultured at 28.5 °C in E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl\(_2\) 0.33 mM MgSO\(_4\) and 0.1% Methylene Blue), unfertilized eggs were removed, and embryos were scored 24 h post-fertilization according to their appearance.

**Microscopy** - Injected Zebrafish embryos were dechorionated, anesthetized with Tricaine (0.016% w/v), and orientated in 3% methyl cellulose on coverslips. Fluorescence was visualized with an LSM510 META inverted confocal microscope (Zeiss, Oberkochen, Germany). Images were captured digitally with a Zeiss MRM AxioCam camera and assembled in Photoshop 8.0 (Adobe Systems, Mountain View, CA). For nuclear staining, embryos were immersed in 1:1000 dilution of TOPRO-3 stain (monomeric cyanine nucleic acid stain; Molecular Probes) for 30 min at room temperature and washed three times with E3. To assess apoptotic cell death, the embryos were additionally immersed in 3 \( \mu \text{g/ml} \) Acridine Orange (acridinium chloride hemi[zinc chloride]; AO; Molecular Probes)
for 15 min and washed three times with E3. For 3D imaging, a stack of 10 frames was collected at an interval of 10-24 µm between adjacent slices, and the images were assembled to obtain a 3D projection. To dissociate the GFP/AO or GFP/YFP fluorescence signals, analysis was performed by emission fingerprinting in confocal lambda mode. A stack of 2-3 frames of the embryonic tail region 24 h post-fertilization was collected at an interval of 20 µm, and the number of AO stained cells was counted to estimate the amount of apoptotic cells. For the time-lapse analysis of the living cells, zebrafish embryos 24 h post-fertilization were oriented in 1.2% low melting agarose (1.2% in E3 with Tricaine 0.09% w/v) and covered with E3. Images taken 24, 28, and 32 hours post-fertilization, were corrected for photobleaching effects, which reduced the signal ~22.4% over the course of the experiment.

Cell fractionation, western blot analysis, and the filter trap assay - Injected embryos were resuspended in cold lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, and protease inhibitor cocktail (Roche) in PBS) and lysed by passing through a 0.6 mm needle. After freezing and thawing the extracts, benzonase was added (75 U), and the extracts were incubated at 4 °C for 1 h with gentle agitation. Extracts were centrifuged at 2,000 x g for 3 min at 4 °C in the presence of glass beads to facilitate separation of the chorion debris. The supernate was collected and protein concentration was determined with a protein assay kit (Bio-Rad). For the detection of Hsp70-YFP (and Q102-GFP in Figure 4b), embryos were first dechorionated and yolk dissected, as previously described (23). For subcellular fractionation, extracts were centrifuged at 100,000 x g for 30 min to obtain the supernatant and pellet fractions. The pellet was washed once with lysis buffer, centrifuged at 100,000 x g for 15 min and either dissolved in SDS sample buffer or 100% formic acid. The latter was incubated for 3 h at 37 °C with agitation, vacuum-dried, and resuspended in SDS-sample buffer, as previously reported (24). Fractions were heated to 95 °C for 5 min and analyzed using SDS-PAGE and Western blotting (anti-GFP, anti-Hsp40, or anti-β-Galactosidase), according to standard procedure. For the filter trap assay, an equal volume of 4% SDS / 100 mM DTT was added to embryo extracts, and the mixture was heated for 5 min at 95°C. Several dilutions between 25 and 1000 µg were filtered through a cellulose acetate membrane (pore size = 0.2 µm), according to protocol (6). Aggregates retained on the filter were detected with an anti-GFP antibody. In all cases, extracts were analyzed in parallel via SDS-PAGE and western analysis to ensure equal expression levels among all conditions. For quantitating the results of western blotting or the filter trap assay, an LAS-3000 imager (Fujifilm Image Reader) was used.

Chemical treatments - Chemicals from the DIVERSet library (313B02, 293G02, and 306H03) were obtained from ChemBridge Corporation (San Diego, CA). Isoproterenol Hydrochloride was dissolved in H2O, the other compounds were dissolved in DMSO, at the indicated concentrations. After the injection, the embryos were randomly split into two groups: one group was transferred into E3 supplemented with 1% DMSO and the indicated concentration of compound; the other group was transferred into E3 supplemented with 1% DMSO as a control. The chorion was carefully disrupted using fine forceps to facilitate penetration of the compound through the chorion, and unfertilized eggs were removed several hours post-fertilization. Embryos were cultured at 28.5 °C for 24 h post-fertilization and extracts were prepared and analyzed as described above. The filter trap assay signal associated with extracts derived from DMSO or H2O treated embryos was set to 100%.

In vitro aggregation reactions - In vitro aggregation reactions were performed essentially as described (12). Briefly, purified GST-Htt exon 1 fusions (3 µM) were mixed with 3 or 30 µM Congo red, 313B02 (3), 293G02 (4), 306H03 (5), or DMSO. To initiate the aggregation reaction, 2.5 U PreScission Protease in buffer A (50 mM Tris-
HCl, pH 7.4; 150 mM KCl) was added. Reactions were incubated at 30 °C for 8 hours and then stopped by adding an equal volume of 4% SDS / 100 mM DTT. The amount of SDS-insoluble aggregates per reaction was analyzed using the filter trap assay (anti-HA), as described above.

Statistical analyses - The data were compared using Bonferroni’s post hoc test and one-way ANOVA (alpha-level was set to 0.05), as indicated.

Docking of compounds to a triangular polyQ dimer - Using the program AutoDock Version 3.0 (25), we simulated the docking of models for the examined compounds (293G02, 306H03, 313B02, Isoproterenol, Mycophenolic acid, PGL-135, and Congo red) to a specific β-helical model for a polyQ prototubir. The structural model of a polyQ prototubir was adopted from the work of Stork et al. (8), who had suggested a left-handed triangular β-helix as the secondary structure motif for polyQ amyloid fibers. More specifically, a dimer of two β-helical polyQ peptides each covering 36 residues was selected. Five structural snapshots of the dimer solvated in water were taken at a temporal distance of 50 ps from the molecular dynamics trajectory, which served for equilibration of the modeled dimer structure with its aqueous environment at room temperature and ambient pressure in the quoted paper. The polyQ dimer snapshots were prepared for the AutoDock simulations using respective AutoDockTools (ADT), which assigned partial charges and solvation parameters to the atoms of the receptor peptides. Structural models for the ligand molecules were generated and partial charges were assigned using the quantumchemistry program MNDO (26). Rotatable bonds within the ligands were defined through ADT. AutoDock applies an interpolation scheme to evaluate the energy of a ligand in the vicinity of a receptor using grid maps that are pre-calculated over the receptor for each atom type in the ligand. We calculated the grid maps with 127 × 127 × 127 points and a grid spacing of 0.375 Å centered at the polyQ dimers using the AutoGrid tool within ADT. For each of the five polyQ dimer snapshots, we simulated the docking of the seven compounds using the Lamarckian Genetic Algorithm available in AutoDock. For each receptor-ligand pair, 100 separate dockings were performed and sorted by ascending free energy. The 20 docking structures of lowest free energy were visualized for each ligand using the software VMD (27).

RESULTS

PolyQ-expanded htt is deposited into inclusions of SDS-insoluble aggregates in D. rerio - We first evaluated whether we could reproduce key features of HD in zebrafish. Embryos at the 1-2 cell stage were injected with mRNA encoding an N-terminal fragment of htt containing 4, 25, or 102 Q fused to green fluorescent protein (Q4-GFP; Q25-GFP; Q102-GFP, respectively). Q4-GFP and Q25-GFP fusion proteins were diffusely distributed, whereas the mutant polyQ-expanded htt fusion protein, Q102-GFP, accumulated in inclusion body-like aggregates throughout most embryos after 24 h (Figure 1A). Higher magnification images of the hatching gland indicated that Q102-GFP, but not the shorter polyQ variants, accumulated in large, cytoplasmic inclusions adjacent to the nuclei (Figure 1, B and C). Analysis of embryos at later time points using time-lapse microscopy showed that Q102-GFP inclusions grew in size by efficient incorporation of soluble Q102-GFP, resulting in its depletion from the cytoplasm (Figure 1D and Supplementary Video 1 online). When total cellular extracts of injected embryos 24 h post-fertilization were subjected to high speed centrifugation, both Q4- and Q25-GFP remained exclusively in the soluble supernatant fraction, whereas a significant portion of Q102-GFP was found in the SDS-insoluble pellet fraction and could only be dissolved by formic acid treatment (Figure 2A) (24). Analysis of total embryo extracts by an established filter trap assay (6) confirmed that Q102-GFP, but not Q4-GFP or Q25-GFP, formed large SDS-insoluble aggregates that
were retained by the 200 nm pore sized membrane (Figure 2B).

**PolyQ length dependent toxicity is independent of detectable inclusions** - To determine whether the polyQ-GFP fusion proteins caused toxicity, we evaluated the phenotypes of embryos expressing Q4-, Q25-, or Q102-GFP, 24 h post-fertilization. Embryos were scored as visibly normal, abnormal, or dead. Q102-GFP expression was associated with increased numbers of dead or morphologically abnormal embryos when compared with Q4- or Q25-GFP expression (Figure 3A). Acidine Orange staining of embryos indicated that Q102-GFP expression caused increased apoptosis (Figure 3B, Supplementary Figure 1 online). Low levels of apoptosis and abnormal phenotypes in embryos expressing Q4-GFP and Q25-GFP were also observed, which was likely due to background toxicity caused by these fusion proteins (Figure 3, A and B). Interestingly, further analyses showed that nearly all (~95 %) of the apoptotic cells were devoid of visible inclusions, which must be > ~0.5 µm in diameter for detection in our experimental setup. On the other hand, almost all cells containing detectable inclusions were non-apoptotic (~99 %), suggesting that pre-fibrillar Q102-GFP may instead be the toxic agent (Figure 3C, top panels). Indeed, apoptotic cells containing diffusely distributed Q102-GFP were detected (Figure 3C, bottom panels). Thus, our results indicate that expression of polyQ-expanded htt is toxic to the zebrafish embryo, but toxicity is not associated with the presence of visible inclusions.

**Hsp70 and Hsp40 suppress both polyQ aggregation and toxicity** - Molecular chaperones are known to modulate both polyQ aggregation and toxicity (28). We next evaluated whether we could reproduce this important feature by testing the effect of the human chaperones Hsp70 and Hsp40 on Q102-GFP aggregation and toxicity in the zebrafish embryo. Co-expression of Hsp40 and Hsp70 fused to YFP (Hsp70-YFP) reduced the amount of SDS-insoluble aggregates of Q102-GFP, as judged by the filter trap assay, while total protein levels of Q102-GFP were not detectably altered (Figure 4, A and B). Fluorescence microscopy revealed that Hsp70-YFP associated with inclusions of Q102-GFP (Figure 4C), suggesting that the chaperone machinery recognized Q102-GFP as an abnormally folded species. Expression of Hsp40 and Hsp70-YFP reduced the number of detectable inclusions (~41.8 % of control, data not shown), apoptotic cells (~80 % of control, data not shown), and dead embryos (Figure 4D), while increasing the number of phenotypically normal embryos expressing Q102-GFP. Thus, aggregation and toxicity associated with Q102-GFP expression in zebrafish is partially suppressed by Hsp40 and Hsp70.

**Identification of compounds that inhibit polyQ aggregation** - We next tested the effect of several classes of compounds on the aggregation of Q102-GFP in the zebrafish embryo (Figure 5). First, we evaluated the capacity of known inhibitors of the polyQ aggregation process to suppress Q102-GFP aggregation in this vertebrate system. PGL-135 and Congo red (16,29) reduced Q102-GFP aggregation in zebrafish, as judged by the filter trap assay (Figure 6A). In contrast, Isoproterenol HCl did not alter Q102-GFP aggregation in the zebrafish embryo, consistent with a recent study in a cell culture model (30). Surprisingly, Mycophenolic acid, a compound known to suppress htt inclusion formation in cell culture (30), had no effect on Q102-GFP aggregation in zebrafish at the maximum tolerated dose. Thus, in the case of PGL-135, Congo red, and Isoproterenol HCl, our results substantiate observations in other HD models. However, in contrast to data obtained in cell culture (30), our findings suggest that Mycophenolic acid is not an effective inhibitor of polyQ aggregation in the vertebrate system.

Next, we set out to identify new compounds that inhibit polyQ aggregation. Considering the hypothesis that polyQ and PrPSc aggregates have similar amyloid core structures (8,31), we tested three compounds (313B02, 293G02, and 306H03) that were
previously identified in in vitro screens for inhibitors of prion propagation (32, pers. comm. A. Giese). All three of these compounds inhibited the aggregation of polyQ-expanded htt in vitro (Figure 6A), indicating these compounds interact directly with mutant htt to reduce its aggregation. However, only 293G02 and 306H03, both derivatives of N'-benzylidene-benzohydrazide (NBB), also inhibited the formation of detergent insoluble aggregates of Q102-GFP in vivo (Figure 6B). Interestingly, 306H03 was a more effective inhibitor of polyQ aggregation than 293G02 in vivo, but not in vitro (Figure 6A and 6B).

To substantiate the effect of NBBs on polyQ aggregation, we set up a similar model of HD in mammalian neuroblastoma cells (N2a). In this system, expression of Q102-GFP, but not Q4-GFP or Q25-GFP, resulted in the formation of large, perinuclear inclusions containing SDS-insoluble aggregates; however, Q102-GFP was not overtly toxic to these cells under the tested conditions (Supplementary Figure 2A and 2B online). Concordant with the results from the zebrafish system, NBBs proved efficient in suppressing Q102-GFP aggregation in N2a cells (Supplementary Figure 2C online). Taken together, these data suggest that NBBs represet a new class of compounds that effectively inhibit polyQ aggregation. Notably, none of the studied compounds suppressed the abnormal morphology and death associated with Q102-GFP expression in zebrafish embryos (data not shown). Thus, the NBBs may interfere with the formation of large SDS-resistant polyQ aggregates downstream of the accumulation of toxic polyQ species.

To gain insight into the effects of the anti-prion compounds on polyQ aggregation, we simulated their docking to a structural model for a polyQ protofibril (8). The simulations predict large binding free energies in a narrow range between 4.8 - 7.2 kcal/mol (Supplementary Table 1 online), indicating thermally stable dockings for all investigated compounds. As exemplified in Figure 7 for the four molecules with largest binding free energies, all compounds were found to preferentially bind to the two ends of the polyQ helices. Well-defined docking positions are predicted for the two NBB compounds (293G02 and 306H03), which both exhibit an amide group perfectly suited for hydrogen bonded attachment to the polyQ peptide backbone. The docking positions of Congo red and 313B02, however, show a larger disorder. All compounds additionally exhibit a variety of further binding interactions with the peptides. In conclusion, our simulations using a polyQ model predict that NBBs specifically bind to the ends of the polyQ helices, a mechanism that would prevent fibril growth. Indeed, other structural models for polyQ protofibrils exist (33), but all are characterized by dangling H-bond attachment sites (i.e. backbone C=O and N-H groups) at the unsaturated ends of β-sheet fibril, which could mediate NBB interaction.

DISCUSSION

We set out to develop a zebrafish HD model system suitable for whole organism validation of candidate compounds. The zebrafish model of HD described in this study allows the deposition of htt aggregates to be monitored in real-time in a living vertebrate organism. Expression of polyQ-expanded htt gave rise to the formation of large, SDS-insoluble inclusions and was associated with enhanced apoptosis, as well as pronounced morphological defects and reduced viability of zebrafish embryos. Interestingly, among the visibly abnormal embryos, a characteristic ‘cyclopic’ phenotype was increasingly observed in embryos expressing Q102-GFP (data not shown). This mutant phenotype is characterized by fusion of the eyefields yielding a single eye in the midline (34) and has recently been found to be associated with defects in Ca\(^{2+}\) homeostasis of the endoplasmic reticulum in zebrafish (35). The observed enhancement of the cyclopic phenotype may therefore reflect an adverse effect of Q102-GFP on Ca\(^{2+}\) homeostasis. Indeed, perturbed Ca\(^{2+}\) homeostasis is hypothesized to be involved in HD
pathogenesis (36). An important observation was that both the aggregation and toxicity of mutant htt was suppressed by the molecular chaperones, Hsp40 and Hsp70, mimicking observations from other HD models (28). Because key features of HD are observed in our zebrafish model, we propose that zebrafish is a valid model organism for identifying factors that influence disease pathogenesis.

The newly established zebrafish model proved useful in evaluating the effectiveness of compounds in inhibiting polyQ aggregation and toxicity in the context of a whole vertebrate organism. Drug absorption into the zebrafish embryo is likely facilitated by its aqueous environment and lack of blood brain barrier at early stages in development; therefore, compounds could be tested for their effect on polyQ aggregation while minimizing complicating factors that impede absorption. The unique feature that drug testing can be performed within two days made this model a cost and time efficient alternative to existing mouse models of HD. The ability to differentiate between effects on polyQ-mediated aggregation versus toxicity is another advantage of the zebrafish system, which is lacking in several other HD models, such as the N2a cell culture model tested in this study (Supplementary Figure 2 online).

The zebrafish model also takes into account the stability and the cellular targeting of compounds in an organism. For example, one of the tested compounds, 313B02, was effective in inhibiting polyQ aggregation in vitro, but was not effective as an aggregation inhibitor in vivo (Figure 6A and B), suggesting that this compound is not stable and/or not targeted efficiently in the zebrafish. Using zebrafish, we were also able to evaluate whether a compound caused side effects when present at active concentrations in a whole organism. For instance, Mycophenolic acid, a compound known to suppress htt inclusion formation in cell culture, caused pronounced apoptosis in the brain region of the zebrafish embryo at these doses (data not shown), and had no effect on polyQ aggregation at the maximum tolerated dose (Figure 6B).

We used the newly established zebrafish model to identify NBBs as effective inhibitors of polyQ aggregation. Two of the analyzed anti-prion compounds, 313B02 and 293G02, were previously identified as inhibitors of the recruitment of prion monomers into PrPSc aggregates (32), while compound 306H03 has since been discovered in a similar screen (A. Giese, pers. comm.). PrPSc and mutant htt are neurodegenerative disease proteins that accumulate in structurally similar fibrillar aggregates that may have a common beta-helical amyloid core structure (8,31). We therefore hypothesized that inhibitors of the aggregation of PrPSc may also be effective in preventing polyQ protein aggregation. Our observation that anti-prion compounds are indeed potent inhibitors of polyQ aggregation suggests that polyQ aggregates and prions share common structural epitopes. Similar results obtained from an N2a neuroblastoma cell culture model of HD (Supplementary Figure 2 online) confirmed our results from zebrafish and further established the zebrafish system as a valid model to study human disease. Taken together, these findings reinforce the view that common mechanisms underlie the pathogenesis of diverse neurodegenerative diseases and suggest that common therapeutic targets may exist.

Based on our results, NBBs may constitute a new class of compounds with “generic” anti-aggregation activity, perhaps preventing the misfolding and aggregation of a wide variety of neurodegenerative disease proteins. NBBs provide a novel and promising lead structure for the development of new therapeutics in several regards. The NBBs used in this study satisfy Lipinski’s ‘rule of 5’ for drug-likeness by having fewer than 5 H-bond donors, 10 H-bond acceptors, a molecular weight smaller than 500, and a calculated Log P (cLogP) smaller than 5, which would predict efficient absorption or permeation (37). Importantly, our data indicate that in a vertebrate organism, NBBs can reach the cytoplasm in active concentrations without causing overt toxic side effects up to the limits of their solubility in water. Furthermore, our analyses indicate that the hydroxyl groups of the NBB compounds, which were shown to be crucial
for anti-prion activity in vitro (32), are not essential for inhibition of polyQ aggregation in vivo. Actually, 293G02, although exhibiting the highest potency in vitro, was a less effective inhibitor of polyQ aggregation than 306H03 in vivo (Figure 6A and 6B, Supplementary Figure 2C online). Most likely, the presence of a chloride group, rather than hydroxyl groups, enhances cell permeability by decreasing the total polar surface area (tPSA) from 81.92 Å² (293G02) to 41.46 Å² (306H03) and increasing the hydrophobicity cLogP from 2.94 (293G02) to 4.67 (306H03). Thus, NBBs such as 306H03 may be developed that are more likely to cross the blood brain barrier efficiently. Presumably, the disease-modifying therapeutic effect of aggregation inhibitors in protein aggregation diseases also depends on the nature of the toxic species and whether toxicity occurs primarily through a loss-of-function or gain-of-function mechanism. New classes of drugs which interfere with aggregate formation at the molecular level such as the NBBs may provide valuable tools to further dissect the molecular steps involved in disease pathogenesis in vivo.

The zebrafish model has also allowed us to investigate the question of whether inclusions of polyQ-expanded htt contribute directly to toxicity in a vertebrate organism. Our observation that the majority of apoptotic cells were devoid of visible inclusions suggests that polyQ toxicity is not primarily caused by detectable inclusions, in line with previous findings in cell culture (11,12). Smaller inclusions/aggregates with a diameter < ~0.5 µm, which would escape detection using our experimental setup, are therefore implicated in causing toxicity in our system. This may explain why NBBs could suppress the formation of SDS-insoluble aggregates without detectably suppressing polyQ toxicity (Figure 6 and data not shown). We suspect that NBBs effectively inhibit primarily later stages of Q102-GFP aggregation. Thus, early soluble intermediates in the htt aggregation pathway associated with toxicity may still accumulate. Future studies aimed at assaying structural derivatives of NBBs may lead to the identification of more effective suppressors of polyQ toxicity.

Large scale screens will continue to identify promising compounds that inhibit aggregation processes in vitro or in cell culture. A systematic approach for validating such candidate compounds should include the judicious use of vertebrate model systems to evaluate efficacy against polyQ aggregation and toxicity before proceeding to clinical trials. Cost and time efficient drug testing using the zebrafish model described here is likely to prove useful for this purpose.
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**FOOTNOTES**

Abbreviations: Huntington’s disease (HD); polyglutamine (polyQ); N’-benzylidene-benzohydrazide (NBB); huntingtin (htt); inclusion bodies (IBs)

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Note: Supplementary information is available on the Journal of Biological Chemistry website.
FIGURE LEGENDS

**Fig. 1.** Q102-GFP forms perinuclear inclusions in *D. rerio*.  
*A*, Fluorescent (*top panels*) and brightfield (*bottom panels*) images of zebrafish embryos expressing Q4-GFP, Q25-GFP, or Q102-GFP, 24 h post-fertilization.  
*Scale bar*, 200 µm.  
*B*, Higher magnification images of polyQ-GFP expression patterns in the embryonic hatching gland.  
*Scale bar*, 10 µm.  
*C*, Embryos expressing Q102-GFP stained with TOPRO (shown in blue) to visualize nuclei in the hatching gland.  
*Scale bar*, 10 µm.  
*D*, The dynamics of Q102-GFP aggregation, as monitored by time-lapse microscopy 24, 28, and 32 h post-fertilization.  
The formation of an aggregate structure (*long arrow*) and the incorporation of soluble Q102-GFP into pre-existing aggregates (*short arrow*) are indicated.  
These images, which were originally associated with a video (Supplementary Video 1 online), have been corrected for photobleaching effects.  
*Scale bar*, 10 µm.

**Fig. 2.** Q102-GFP forms SDS-insoluble aggregates in *D. rerio*.  
*A*, Analysis of the solubility of the polyQ-GFP fusions.  
Extracts from embryos (24 h post-fertilization) expressing Q4-GFP, Q25-GFP, or Q102-GFP were subjected to high speed centrifugation, and the resulting fractions were analyzed by SDS-PAGE and western blotting with anti-GFP antibody.  
*T*, total cell lysate;  
*S*, supernate;  
*P*, pellet fraction dissolved in SDS;  
*FA*, pellet fraction dissolved in formic acid.  
*Asterisk*, free GFP cleaved from the fusion protein.  
*B*, Filter trap analysis of polyQ-GFP fusions.  
Increasing amounts (25 µg, 50 µg and 100 µg) of total cell extracts from *A* were analyzed by filter trap assay in the presence (*bottom row*) and absence (*top row*) of SDS/DTT at 95°C.

**Fig. 3.** PolyQ length-dependent toxicity is independent of visible inclusions.  
*A*, Embryos were injected at the 1-2 cell stage with mRNA encoding the indicated GFP fusions.  
Normal (wt), abnormal, and dead embryos were counted 24 h post-fertilization.  
Data are mean values of n=12-20 experiments ± S.D.  
*, *p* < 0.01 versus Q4-GFP or Q25-GFP injected embryos (Bonferroni’s post hoc test).  
*B*, Fluorescent images of Acridine Orange stained embryos expressing the indicated polyQ-GFP fusions 24 h post-fertilization and subsequent quantification of apoptotic cells.  
As a negative control, uninjected embryos expressing no polyQ-GFP fusion were also analyzed (*Uninj.*).  
*Scale bar*, 100 µm.  
Data are mean values of n=3-4 experiments ± S.D.  
*C*, Apoptotic cells are largely devoid of visible inclusions.  
*Top panels*, confocal images demonstrating that aggregate structures containing Q102-GFP are in the majority of cases not detectable in apoptotic cells.  
Embryos were prepared as described in *B*.  
*Scale bar*, 20 µm.  
*Bottom panels*, higher magnification images of the embryonic hatching gland showing an apoptotic cell with diffusely distributed Q102-GFP.  
Neighboring cells containing aggregates are not apoptotic.  
*Scale bar*, 10 µm.  
Quantitation of apoptotic cells with and without inclusions, as well as quantitation of cells containing inclusions that are apoptotic verses non-apoptotic is also shown.  
Data are mean values of n=3 experiments ± S.D.

**Fig. 4.** Hsp40 and Hsp70-YFP suppress polyQ aggregation and toxicity.  
*A*, Filter trap analysis of total extracts derived from embryos injected with Q102-GFP mRNA together with β-Galactosidase mRNA (control) or Hsp40 and Hsp70-YFP mRNA at the 1-2 cell stage.  
Total SDS-insoluble aggregates trapped on the membrane were detected with anti-GFP and quantified.  
Signal intensity resulting from the co-injection of Q102-GFP mRNA with β-Galactosidase mRNA was defined as 100%.  
*B*, Western blot analysis of total extracts from *A* to detect β-Galactosidase, Hsp40, Hsp70-YFP, and SDS-soluble Q102-GFP.  
*C*, Confocal images showing that Hsp70-YFP colocalizes with Q102-GFP inclusions, as assessed by emission fingerprinting.  
*Scale bar*, 20 µm.  
*D*, Quantitation of normal (wt), abnormal, and dead embryos 24 h post-fertilization, after injection at the 1-2 cell stage with Q102-GFP and β-Galactosidase or
Hsp40/Hsp70-YFP mRNA. Data are mean values of n=20 experiments ± S.D. * $p < 0.01$ versus Q102-GFP/β-Galactosidase injected embryos (ANOVA).

**Fig. 5.** Molecular structures of the compounds tested.

**Fig. 6.** N'-benzyldiene-benzohydrazides inhibit Q102-GFP aggregation *in vitro* and in *D. rerio*.

*A*, Effect of the indicated chemical compounds on HA-HttEx1Q53 aggregation *in vitro*, as assessed by the filter trap assay (anti-HA) with quantitation. The signal intensity from the control sample containing solvent alone was set to 100%. Data are mean values of n=3 experiments ± S.D. *B*, Filter trap analysis of extracts derived from embryos expressing Q102-GFP in the presence and absence of the indicated compounds. Embryos expressing Q102-GFP were treated for 24 h with DMSO or H₂O (solvent), 30 µM PGL-135, 100 µM Congo red, 100 µM 313B02, 50 µM 293G02, 50 µM 306H03, 1 µM Mycophenolic acid, or 10 µM Isoproterenol HCl. Extracts of embryos were analyzed using the filter trap assay (anti-GFP), followed by densitometric quantitation. The signal intensity from controls subjected to solvent alone was set to 100%. The data are mean values of n=3-5 independent injections ± S.D.

**Fig. 7.** Docking of compounds to a polyQ protofibril model, comprising a dimer of 36Q peptides arranged in a two-coiled triangular β-helical structure (8).

The protofibril would grow by attachment of further coils at the top and bottom ends of the β-helix. The polypeptide backbones of the polyQ dimers are drawn in tube representations, and the docked compounds are shown in line representations. The five different polyQ snapshots and the associated dockings are distinguished by color coding. For each of the four ligands (Congo red, 313B02, 293G02, 306H03) and each snapshot, the 20 dockings with lowest free energies are shown. Additionally, the average value of these free energies is given for each of the four selected compounds.
Figure 2

A

| kDa | T | S | P | FA | T | S | P | FA |
|-----|---|---|---|----|---|---|---|----|
| 80  |   |   |   |    |   |   |   |    |
| 47  |   |   |   |    |   |   |   |    |
| 32  |   |   |   |    |   |   |   |    |

B

| SDS no SDS | Q4-GFP | Q25-GFP | Q102-GFP |
|------------|--------|---------|----------|
|            |        |         |          |
|            |        |         |          |
|            |        |         |          |
|            |        |         |          |
Figure 3

A

Total embryos (%)

wt  |  abnormal  |  dead

Q4-GFP  |  Q25-GFP  |  Q102-GFP

B

Uninjected  |  Q4-GFP  |  Q25-GFP  |  Q102-GFP

Apoptotic cells (% of control)

0  |  200  |  400  |  800

Uninj.  |  Q4-GFP  |  Q25-GFP  |  Q102-GFP

C

GFP  |  AO  |  Merge

Total apoptotic cells (%)

0  |  20  |  40  |  80  |  100

GFP  |  AO  |  Merge

Total cells containing inclusions (%)

0  |  20  |  40  |  60  |  80  |  100

non-apoptotic  |  apoptotic
**Figure 4**

**A**
- Bar graph showing Q102-GFP aggregates (% of control) for Q102-GFP/β-Gal and Q102-GFP/Hsp40/Hsp70-YFP.

**B**
- Table showing the expression levels of β-Gal, Hsp70-YFP, Hsp40, and Q102-GFP under different conditions.

**C**
- Images showing GFP, YFP, and Merge for Q102-GFP, Hsp40, and Hsp70-YFP.

**D**
- Bar graph showing total embryos (%) for different conditions: wt, abnormal, and dead, with Q102-GFP/β-Gal and Q102-GFP/Hsp40/Hsp70-YFP.
Figure 5

- PGL-135
- Congo red
- 313B02
- 293G02
- 306H03
- Mycophenolic acid
- Isoproterenol HCl
Figure 6

A

| Compound       | Concentration |
|----------------|---------------|
| DMSO           | 3 μM, 30 μM  |
| Congo red      | 3 μM, 30 μM  |
| 313B02         | 3 μM, 30 μM  |
| 293G02         | 3 μM, 30 μM  |
| 306H03         | 3 μM, 30 μM  |

B

Solvent Compound

- PGL-135 (30 μM)
- Congo red (100 μM)
- 313B02 (100 μM)
- 293G02 (50 μM)
- 306H03 (50 μM)
- Mycophenolic acid (1 μM)
- Isoproterenol HCl (10 μM)
Congo red
$\Delta G = -7.2 \text{ kcal/mol}$

293G02
$\Delta G = -6.4 \text{ kcal/mol}$

313B02
$\Delta G = -6.7 \text{ kcal/mol}$

293G02
$\Delta G = -6.4 \text{ kcal/mol}$

306H03
$\Delta G = -6.2 \text{ kcal/mol}$
Identification of anti-prion compounds as efficient inhibitors of polyglutamine protein aggregation in a zebrafish model

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