Spring 2003

Structure-Activity Relationships Inhibitors of Polyglutamine

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College: Arts + Sciences  Department: BCMB
Faculty Mentor: Ronald B. Wetzel

PROJECT TITLE: Structure-Activity Relationships of Inhibitors of Polyglutamine Aggregation

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Ronald Wetzel, Faculty Mentor
Date: July 25, 2003

General Assessment - please provide a short paragraph that highlights the most significant features of the project.

Comments (Optional):
Structure-Activity Relationships of Inhibitors of Polyglutamine Aggregation

Erin Newby

Senior Honors Project

August 2003
ABSTRACT

The presence of an expanded polyglutamine (polyGln) domain in the huntingtin protein underlies the pathogenic events in Huntington’s disease (HD), a neurodegenerative disorder for which no cure exists. The ability of the expanded polyGln domain to form insoluble aggregates appears to be critical in the HD pathogenesis, indicating that polyGln aggregation is a potential target in the development for a cure.

Wetzel and colleagues have established a highly sensitive, fast, reproducible and specific polyGln aggregation assay capable of following the ability of very low amounts of polyGln aggregates to recruit additional polyGln peptides. Sub-micromolar concentrations of synthetic polyGln aggregates are coated on a 96-well plate and are incubated for different times with nanomolar amounts of biotinylated polyGln peptide. Europium-streptavidin addition and time-resolved fluorescence allow them to quantify the amount in femtomoles of polyGln peptide incorporated into aggregates. They refined this microtiter plate assay as a screening assay for aggregation inhibitors. In screening application, the assay can detect with reproducibility a 100 µM compound inhibiting a polyGln aggregate extension reaction by a factor of 2 or more (i.e. ≥ 50% inhibition).

Of over one thousand compounds of the NINDS (National Institute of Neurodegenerative Disorders and Stroke) library tested with the microtiter plate assay, nine compounds were revealed as potential inhibitors of polyGln aggregation and their IC₅₀ values were determined. Interestingly, when other groups used different screening assays (worm model, cell based assay, etc.) to test the NINDS library, they identified some of these hits and/or other compounds. In this project, we propose to study the structures of the nine hits identified by Wetzel’s laboratory and to compare them to the database of all compounds tested for compounds with structural similarities. If we find compounds with similar structures, we will determine their IC₅₀ value. These results will help us in the determination of requisite chemical groups for inhibiting polyGln aggregation. We also propose to look at the hits of the other groups and to compare them to these nine potential polyGln aggregation inhibitors to determine if compounds with common structures can act as inhibitors for different targets. These structure-activity relationship studies are important for defining potential therapeutic agents and serve as a starting point for designing and synthesizing more active analogs.
INTRODUCTION

1 - Clinical Symptoms

Huntington's disease (HD) is a rare disease of hereditary transmission that only affects about 0.01% of the population. The initial manifestations are often behavioral and the diagnosis may not be evident for months or years until the classical movement disorder develops. The prominent characteristics of the disease include chorea, defined as “random purposeless involuntary movement,” and bradykinesia, or abnormally slow movement, which is often seen in the early stages (1). The choreiform movement disorder is progressive, affects the extremities and face, and it is associated with slowness and clumsiness of fine movements and postural instabilities. In the late stages of the disease, dystonia sets in the muscles in the face, neck, or limbs, which contract indefinitely, leaving the person rigid and bedridden. These movements are due to neuronal dysfunction and loss. Post-mortem examination of HD patients show significant shrinkage of the brain that can be up to 20% in advanced cases (2). Onset of the disease usually occurs between 35 and 42 years of age, and affected individuals usually die about 17 years after onset (2).

No cure exists for HD patients. Dopamine antagonists, which balance out the direct and indirect neuronal pathways involved in controlling motor function, may be administered to patients to relieve the symptoms of chorea. However, as the striatal output neurons die, the dopamine antagonists become less effective in suppressing choreic movement (2). Antidepressants can also be prescribed to alleviate emotional and behavioral symptoms, but this medication possesses the side effect of increasing bradykinesia.
2 - Molecular aspects

2.1 - Mutant huntingtin and aggregation

In 1993, an international group of scientists identified the mutation in the gene interesting transcript 15 (IT15) responsible for the disease (3). The gene IT15 is composed of 67 exons and encodes a protein of 3,144 amino acids, called huntingtin. Exon 1 contains a CAG trinucleotide repeat that encodes the amino acid glutamine. In healthy individuals the number of CAG repeats, i.e. polyglutamine length is 10 to 34, whereas in HD patients the number increases to 40 or more. In juvenile cases, the number is generally higher than 55 repeats and the mutant gene generally comes from the father. Studies show that huntingtin is expressed in the cytoplasm of most cells in the body. In the brain, huntingtin is found predominantly in the cytosol of neuronal cells and seems to play a vital role in development, as knockout mice do not survive beyond the eighth day of embryogenesis. Curiously, mutant huntingtin that contains an expanded polyglutamine (polyGln) sequence is not only found in the cytoplasm but also in the nucleus, where it tends to aggregate and form inclusions in the neurons of HD patients (4). This same phenomenon has been observed in yeast and transgenic animals (5,6,7). The trend concerning these stretches indicates that the more glutamine (Gln) repeats in the gene, the earlier the onset and the more severe the symptoms. Nine other polyGln disorders similar to HD have been identified, including Kennedy’s disease and the spinocerebellar ataxias types 1, 2, 3 (also known as Machado-Joseph disease), 6, 7, and 17. In each of these disorders, the disease is dependent on increased polyGln length in a particular protein that consequently forms insoluble aggregates. Neuronal loss is noted in all of the polyGln disorders, with each disease targeting specific areas of the brain. The repeated presence of aggregates and neuronal death suggests that the aggregates are involved in the pathology of the polyGln diseases.
One hypothesis as to the reason for aggregation was proposed by Max Perutz and colleagues in a paper concerning the secondary structure of the mutant protein (8). Building on the newfound discovery that HD exists in patients expressing mutant huntingtin protein in excess of 41 glutamine residues, Perutz and colleagues created a computer-generated model demonstrating that the secondary structure of the polyGln aggregate consists of antiparallel β-strands. Perutz presented the idea that the strands form hairpin structures that associate with one another through hydrogen bonding in the aggregation process. The team used this model to synthesize a polyGln peptide and added charged amino acids to each terminus to make the protein more soluble in an acidic environment. At neutral pH, the protein precipitated into aggregates, which were analyzed by several methods to elucidate the secondary structure. Circular dichroism as well as X-ray diffraction confirmed a structure of β-pleated sheets, although the possibility of hairpins could not be explored.

Once the polyGln β-pleated sheets have formed, they accumulate into an inclusion body and aggregate formation occurs. Studies by Scherzinger and colleagues involving glutathione-S-transferase-HD exon 1 fusion proteins show that insoluble huntingtin aggregates require a nucleus or "seed" and a critical concentration of protein to begin the aggregation process (9). An analysis of the aggregation kinetics by Chen and colleagues confirmed the proposal that aggregation occurs by nucleated growth polymerization, and the nucleus was found to be monomeric (12). This monomer is thought to exist as a highly ordered β-sheet structure, and the aggregated β-sheets have been proposed to assume a more ordered, fibrillar, tertiary structure(12). Among the many models for amyloid fibril structure is one proposed by Perutz and colleagues in one of Perutz’s last papers (13). As illustrated in a review by Wetzel, evidence
supports the model of a β-helical structure, which displays greater stability than the β-sheets themselves (14).

2.2 – Recruitment: the aggregation process

As a “seed” recruits mutant huntingtin to form an aggregate, other proteins may be sequestered in the aggregate that are vital to cell function. The fibrils’ resistance to protease activity prevents the release of these functional proteins from the aggregate and the lack of essential proteins disrupts the cell’s response to stress. Ross and colleagues found that in HD cell-culture models, HD transgenic mice, and human HD post-mortem brains, nuclear CBP levels drop in correlation with the detection of CBP in intranuclear polyGln aggregates (17). The lack of CBP leads to dysregulation of CBP-activated transcription, which may account for the toxicity of aggregates localized in the nucleus (18). Other proteins have also been found in intracellular aggregates, including the molecular chaperones Hsp70 and Hsp40, along with the proteasome components ubiquitin and the 20s and 19s complexes (18). The expression of Hsp40 and Hsp70 suggests that the cell recognizes the polyGln aggregates as misfolded conformers and that the cell then upregulates the transcription of the heat shock protein genes in an attempt to stop further polymerization. Increased expression of these heat shock proteins has been shown to suppress the neurotoxicity caused by polyGln aggregates in transgenic animal models (19, 20), even though intracellular inclusions are still present. Muchowski and colleagues found that the inclusions contain Hsp40 and Hsp70 bound to soluble aggregates, which may be subject to protease activity (21). However, when the cell has aged significantly, the production of these chaperones decreases. Hsp40 and Hsp70 continue to associate with mutant huntingtin at a constant rate, leading to chaperone depletion within the cell. The mutant protein concentration is then high enough to overcome these quality-control mechanisms and form ordered fibrils (18).
Additional misfolding by other cellular proteins may also result in neutral or damaging effects due to the absence of chaperones throughout the cell.

2.3 - Toxicity of the polyGln aggregates

Experiments show that the expanded tract has a toxic quality as the expression of extended polyGln tracts, either alone or in the context of a larger protein, causes symptoms of neurological dysfunction in mice (11). Amyloid-like fibrils have been observed in models of expanded CAG repeat diseases such as transgenic mice, *Drosophila, C. elegans*, and yeast, and in HD patient brains (10). In each of the models as well as in HD patients, a noticeable retardation of movement and general neurological dysfunction is observed. Because these effects are always found in correlation with the presence of insoluble aggregates, some aggregated form of polyGln may be essential to the neuronal destruction observed in HD. Although the recruitment mechanism and sequestration of essential proteins have been presented as possible means through which aggregates affect cell death, other hypotheses are still under investigation. Among these hypotheses is the idea that the inclusion bodies of accumulated fibrils interfere with normal cell function and induce apoptosis (2).

3 - The search for a cure

Because polyGln aggregates have been observed repeatedly in correlation with neuronal loss, aggregates are implicated in the progression of the disease. The current theory in a number of laboratories is that the reduction or prevention of mutant huntingtin aggregation will lead to the development of a cure. Several assays have been designed to find molecules that can inhibit polyGln aggregation. Heiser *et al.* studied the effects of antibodies on aggregation, specifically the monoclonal antibody 1C2 that successfully prevented the formation of fibrils *in vitro* (15).
As a result of the added antibodies, soluble aggregates containing protein and antibodies were found, and these aggregates were vulnerable to protease activity. This effect suggests that the antibody works as a chaperone to stabilize the native conformation of the expanded polyGln tract and inhibit nucleus formation (15). Another potential inhibitor of aggregation is an oligonucleotide, as explored in the work of Nellemann et al (16). Realizing the importance of a critical concentration of protein necessary to begin aggregation, Nellemann used antisense oligodeoxynucleotides to downregulate the expression of mutant huntingtin. Although this process proved effective in vitro in the context of PC-12 cells, the oligodeoxynucleotides were instable in vivo and showed difficulty passing the blood-brain barrier (16). In other attempts to prevent aggregation, a wide variety of chemical compounds, including hormones, antidepressants, antibiotics, and potential inhibitors of β-amyloid formation are being tested for inhibition activity.

A library of 1000-plus compounds with pharmacological properties such as antidepressants, hormones, and antibiotics was compiled by the National Institute for Neurological Disorders and Stroke (NINDS) and made available to research laboratories for blind studies. Teams from various labs tested these compounds in the context of cell-based, animal model, and synthetic assays to find inhibition values for cell toxicity, animal responses, and polyGln aggregate extension. The compounds deemed most successful in each assay, or “hits,” were reported to the NINDS, and the compounds’ chemical natures were revealed to the labs.

Due to the vast number of molecules to be tested for inhibition capability, assays known as high throughput screening assays have been developed to examine large quantities of molecules in a short amount of time. Wetzel and colleagues have designed such a screening
assay using synthetic peptides to test the recruiting ability of polyGln aggregates (22). Nanomolar amounts of biotinylated peptide are added to aggregates in a microtiter plate, which is incubated for a period of time to allow extension of the aggregate. The amount of peptide incorporated into the aggregate can be determined with the addition of a europium-streptavidin solution, and a microtiter plate reader detects the fluorescent europium. If a chemical compound is added along with the peptide, one can detect the compound’s ability to inhibit polyGln extension by comparing the amount of peptide integrated in the aggregate to the amount that integrated without the presence of the compound. Previously, in participation with the NINDS study, compounds from the NINDS library were screened using this method. Nine compounds exhibited greater than 50% inhibition at 100 μM (V. Berthelier & R. Wetzel, unpublished results), and these compounds were further tested to determine their IC$_{50}$ values.

To determine which chemical groups confer an inhibition activity, we inspected the database of all the compounds tested for structures similar to these nine hits. We conducted dose-response analysis of structurally related non-hits to quantify weak activities. In order to explore the possibility that some chemical groups may have inhibitory effects on more than one target, we compared our nine hits to hits found in the different types of assays testing for polyGln aggregation and/or toxicity. In comparing our hits to the hits reported by other labs, we were looking for structural similarities to highlight compounds that were possibly overlooked in the screening process because the inhibition values were just below the minimum standard of 50% inhibition. We also correlated the effects of compounds in vitro (in the extension assay) to the effects noted in vivo in a C. elegans model of polyGln disease.
MATERIALS AND METHODS

1 - General Materials

PolyGln peptides were obtained from the Keck Center at Yale University, where custom solid-phase synthesis was used to construct peptides according to the design K2QnK2. The structures and purities of the peptides were confirmed by mass spectrometry at the Keck Center. Biotinylated polyGln peptides were prepared by N-terminal derivatization during solid-phase synthesis.

2 - Purification and preparation of biotinylated polyGln peptides

Established methods were used to prepare the biotinylated peptides and aggregates (22). Biotinyl K2Q28K2 peptides were dissolved at 0.5 mg/ml in a mixture of 50% trifluoroacetic acid and 50%1,1,1,3,3-hexafluoro-2-propanol (TFA/HFIP), agitated, and incubated at room temperature for 30 minutes. After evaporating the volatile solvents under a stream of argon in a fume hood, the peptides were dissolved in H2O/TFA, at a pH of 3, to 0.5 mg/ml. Reverse-phase high-performance liquid chromatography (HPLC) was used to determine the exact peptide concentration, by comparing the peak area at 215 nm to a previously established standard curve of K2Q15K2. The biotinylated peptide was diluted to 10 nM in extension buffer, aliquoted, snap-frozen, and stored at -80°C. Peptides are prepared fresh each month for use in the IC50 assay.

3 - Preparation of polyGln aggregates

The dissolved K2Q28K2 peptide obtained from HPLC was adjusted to 10 μM and 10X PBS added to raise the pH to 7.4. This peptide solution was then incubated at 37°C for 24 hours, followed by incubation at -20°C for 24 hours. The aggregates formed during incubation were characterized by Thioflavin T fluorescence and light scattering and were then centrifuged at
20,800g for 30 minutes at 4°C. The collected aggregates were resuspended in extension buffer and aliquoted into Eppendorf tubes, which were snap-frozen and stored at -80°C.

4 - Preparation of aggregate plates

PolyGln aggregates were fixed to 96-well microtiter plates by adsorption. Each microplate was coated with aggregates diluted in extension buffer to 40 ng per well and incubated, uncovered, at 37°C for 17 hours. Plates were used immediately following this incubation period.

5 - The IC50 assay

A freshly prepared 0.3% gelatin solution was added to a coated microplate, at 100 µl per well, and the plate was incubated for 1 hour at 37°C. The gelatin solution was removed from the plate by washing the wells three times with extension buffer. Biotinylated peptide was then added to the plate at a concentration of 10 nM per well. In the IC50 assay, a serial dilution ranging from 1.5625 to 200 µM is made of each compound, and each dilution is added in triplicate to the plate with the aggregates and peptide. A streptavidin-europium complex is then added, and after an incubation period, the excess is washed out. The bound europium (Eu3+) is released by addition of a chelation buffer, and the released Eu3+ counted by time-resolved fluorescence, which allows us to determine the amount of biotinylated peptide that added to the aggregate during the incubation period. By comparing the Eu3+ incorporation in the presence of an inhibitor to incorporation in its absence, inhibition values of the compounds at each concentration are calculated and used to create a dose response curve. The concentration of potential inhibitor at which the aggregate elongation is inhibited by 50% is recorded as the IC50 value.
RESULTS AND DISCUSSION

1 - Structure-activity relationships of NINDS hits

From previous screening assays, nine compounds, or "hits", were identified that inhibited greater than 50% at 100 μM concentration. In reviewing the structures of the nine hits (Figure 1), we found that some structural features were repeated such as benzene rings with cis hydroxyl group substitutions, β-lactam rings, isolated phenol groups, and quinones. Using these structures as criteria, the database was searched for similar compound structures that might be expected to also exhibit inhibitory activity.

We compiled six groups of additional compounds from the database chosen to represent certain structural features. These groups of structures included benzene rings with cis hydroxyl groups, quinones, β-lactam rings and isolated phenol rings, large multi-faceted ring structures, methacycline derivatives, and steroid hormones.

One common feature suggested by this exercise that might be related to inhibition activity is a phenol group, which might play a role in binding interactions with the aggregate. We chose several compounds from the library that contained this group, including the previous hit moxalactam disodium, as well as the compounds isoxsuprine hydrochloride and cefoperazone sodium (Figure 2). We completed microplate assays to determine the IC₅₀ values for isoxsuprine and cefoperazone, and as indicated in Table 1, these values were found to be 198 and 296 μM respectively. These IC₅₀ values are rather high in comparison to the IC₅₀ of moxalactam disodium determined previously to be 1 μM. (However, a repeat analysis of fresh moxalactam disodium revealed an IC₅₀ of 398 μM; the reason for this discrepancy is unclear.) The phenol hydroxyl group may bind to a portion of the aggregate to disrupt the stability of the hydrogen
bonds in the aggregate structure, or the hydroxyl group may bind to glutamine’s polar side chain to prevent further hydrogen bonding with other peptides in the aggregate.

Another phenolic compound selected from the database was tyrothricin (Figure 2). We found that this compound exhibits an IC\textsubscript{50} of 272 \textmu M. The size of the ring itself may act as an obstruction for additional binding of peptides; alternatively, its activity may be limited to its phenol moiety.

2 – Comparison of the nine hits with hits of other assays

Compounds with structural similarity to our nine hits were also found in hits reported to the NINDS by other laboratories in the consortium. These included lysyl-tyrosyl-lysine acetate and meclocycline sulfosalicylate. We determined the IC\textsubscript{50} values for these compounds (Table 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoxsuprine HCl</td>
<td>198 \textmu M</td>
</tr>
<tr>
<td>Tyrothricin</td>
<td>272 \textmu M</td>
</tr>
<tr>
<td>Cefoperazone Na</td>
<td>296 \textmu M</td>
</tr>
<tr>
<td>Lysyl-tyrosyl-lysine Acetate</td>
<td>330 \textmu M</td>
</tr>
<tr>
<td>Meclocycline Sulfosalicylate</td>
<td>No effect</td>
</tr>
</tbody>
</table>

Lysyl-tyrosyl-lysine acetate (Figure 2) contains a phenol group in analogy to moxalactam disodium, isoxsuprine hydrochloride, and cefoperazone sodium. The IC\textsubscript{50} value found for lysyl-tyrosyl-lysine acetate is also comparable to the values for these other compounds containing a phenol group, suggesting that the structure plays a similar role in inhibiting aggregate elongation in each of these compounds.

Meclocycline differs from methacycline, which has an IC\textsubscript{50} value of 123 \textmu M, by only a chlorine atom substituted in place of a double-bonded CH\textsubscript{2} group (Figure 3). Based on this structural similarity, we believed that meclocycline’s activity would equal that of methacycline; however, our results show no effect by meclocycline up to a concentration of 200 \textmu M. This may be due to the interaction or interference of the counterion sulfosalicylate; alternatively, the additional chlorine atom may destabilize the compound by drawing electrons from the oxygen.
atom in the hydroxyl group on the opposite side of the benzyl ring, making the compound more acidic. This added quality may disrupt the type of interactions with the aggregate that makes methacycline a good inhibitor.

3 – Comparison with hits from an in vivo assay

Among the lab teams who tested the compounds of the NINDS library was a group led by Dr. Christian Neri. Neri and his colleagues used a Caenorhabditis elegans model in which the mutant protein is expressed in the worm. When the affected worms are prodded by a small rod, little to no response is observed, compared to the reflexive retraction observed in normal worms. Neri developed a screening assay to determine the effects of compounds on the aggregation process by growing the worms in microplate wells containing a known concentration of the compounds. The worms mature in about 72 hours, at which point they are placed under the microscope and their physical responses observed as they are prodded with the rod. Recovery of a retractive response indicates the compound’s effect in suppressing the disease state, which may be due to its effect on polyGln aggregation. The compounds are then ranked according to their EC$_{50}$ values, or the concentration of the compound necessary to achieve 50% of the observed effect. Among the most effective compounds in Neri’s assay are those listed in Table 2.

In order to compare our assay results to those of Neri’s group, we completed assays to find IC$_{50}$ values for the group of compounds that exhibit the highest activity in the in vivo assay (Table 2). We chose the 13 compounds considered most potent with C. elegans (C. Neri, personal communication) and upon inspection of these 13 compounds, we immediately recognized one hit from our own assay, protoporphyrin IX. We then completed dose response curves for the remaining 12 compounds and found additional structures that exhibited inhibition
activity in the extension assay (Figure 5). As determined in previous studies (V. Berthelie & R. Wetzel, unpublished), the quinones such as ellagic acid tend to show good IC\textsubscript{50} values, as confirmed by the value for quinalizarin of 91 \textmu M (Figure 4). This earlier study also contrasted oxidized quinone compounds with the dihydroxyquinone form and found that the reduced form of the compounds was less effective in inhibiting elongation of the aggregates. Compounds with hydroxyl groups in cis formation on a benzyl ring were also hypothesized to be more effective than compounds with cis hydroxyl groups on a ring other than benzene, and the high IC\textsubscript{50} value determined for naringin (Figure 4) of 7.8 mM in comparison to the IC\textsubscript{50} values for ellagic acid and quinalizarin, 48 and 91 \textmu M respectively, supports this theory. In addition, the phenol group in naringin does not appear to play a role in the compound's activity such as that of the other phenolic compounds studied.

Of the 12 compounds active in \textit{C. elegans} that we tested, the best IC\textsubscript{50} value obtained in the microplate assay was for the antibiotic cephalosporin C, which contains a \beta-lactam ring and two carboxyl groups (Figure 5). As noted above, another effective compound consisting of a \beta-lactam ring and carbonyl groups in cis formation on a substituted benzene ring is cefoperazone sodium (IC\textsubscript{50} = 296 \textmu M). In previous hits observed in the extension assay (Figure 1), moxalactam disodium was among the best inhibitors when the compound was stored for a period

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50}</th>
<th>EC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoporphyrin IX</td>
<td>12 \textmu M</td>
<td>0.005 \textmu M</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>79 \textmu M</td>
<td>2.000 \textmu M</td>
</tr>
<tr>
<td>Quinalizarin</td>
<td>91 \textmu M</td>
<td>2.800 \textmu M</td>
</tr>
<tr>
<td>Perphenazine</td>
<td>125 \textmu M</td>
<td>1.000 \textmu M</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>133 \textmu M</td>
<td>1.800 \textmu M</td>
</tr>
<tr>
<td>Oxolinic Acid</td>
<td>321 \textmu M</td>
<td>2.300 \textmu M</td>
</tr>
<tr>
<td>Ebselen</td>
<td>408 \textmu M</td>
<td>0.200 \textmu M</td>
</tr>
<tr>
<td>Cannabidiol</td>
<td>743 \textmu M</td>
<td>0.020 \textmu M</td>
</tr>
<tr>
<td>Naringin</td>
<td>7.8 mM</td>
<td>0.800 \textmu M</td>
</tr>
<tr>
<td>MCI-186</td>
<td>252 mM</td>
<td>0.016 mM</td>
</tr>
<tr>
<td>Celastrol</td>
<td>No effect</td>
<td>0.400 \textmu M</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>No effect</td>
<td>2.300 \textmu M</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>No effect</td>
<td>0.900 \textmu M</td>
</tr>
</tbody>
</table>
of time (IC$_{50}$ = 1 μM), making the β-lactam ring, or possibly the decomposition products of the β-lactam ring, a structure of interest.

Other *C. elegans*-active compounds that exhibited noticeable activity in the *in vitro* assay include nortriptyline and perphenazine (Figure 6). The structures of these two compounds are analogous to the structure of the hit hycanthone found by the Wetzel group (Figure 1). Hycanthone, perphenazine, and nortriptyline all have hydrophobic, aromatic groups containing an aminoalkyl appendage that, when protonated in aqueous solution at neutral pH, would be expected to have the properties of ionic detergents. The structure of oxolinic acid (Figure 6) also resembles hycanthone, except that the aminoalkyl appendage is shorter. This difference may make oxolinic acid a weaker ionic detergent and consequently a weaker inhibitor of polyGln aggregation, as shown by the higher IC$_{50}$ value (321 μM) in comparison to the IC$_{50}$ values of hycanthone, nortriptyline, and perphenazine (50 μM, 133 μM, and 125 μM). A possibility for further confirmation of the activity of this ionic detergent structure is testing the other compounds in the NINDS library that are similar to nortriptyline.

The structures of ebselen and MCI-186 are unlike the previous hits of the Wetzel group. What makes these two compounds effective in interaction with the polyGln aggregates is unclear. Both ebselen and MCI-186 were among Neri’s most active compounds (Table 2) but gave only rather unimpressive IC$_{50}$ values in the microtiter plate assay. It is possible that metabolic processing in the worm may enhance the activity of these compounds *in vivo*.

Among the less reactive and ineffective compounds are celastrol, terfenadine, and glibenclamide. The lack of activity by celastrol was not surprising, as past attempts at finding an active steroid ring structure have proven fruitless, with the exception of ethinyl estradiol. As in
the cases of ebselen and MCI-186, the structures for terfenadine and glibenclamide do not possess any of the functional groups present in the Wetzel group’s hits.

CONCLUSIONS

The information gained from this study does not reveal any strong relationship between the in vitro and in vivo assays, as illustrated by a graph (Figure 7) constructed to relate the IC$_{50}$ and EC$_{50}$ values of the top 8 compounds (protoporphyrin IX to cannabidiol). A more definitive test, however, would be to determine dose response curves and IC$_{50}$ values for all 1000-plus compounds in the NINDS collection. It may be that there is a statistically significant overlap among the compounds active in these two assays.

Our studies reveal the potential for activity by compounds containing a β-lactam ring, with the emphasis on the fact that the decomposition products may be the source of the compounds’ activity. A project that could possibly confirm this idea would be one in which a collection of β-lactam antibiotics are obtained, tested to determine the IC$_{50}$ values, and then oxidized. The compounds should be tested again to look for a gain of activity. Another possible follow-up study could be a series of assays to determine the activity of hydrophobic structures resembling perphenazine and nortriptyline. The IC$_{50}$ values that we determined for these compounds are close to the values found for the hits ethinyl estradiol and methacycline in previous studies.
References

Figure 1. The values listed for each compound include 1) the IC\textsubscript{50} value obtained from assays performed using the compound received from NINDS, and 2) the IC\textsubscript{50} value determined from follow-up assays completed using the same compounds obtained commercially. These values can be viewed as a comparison between compounds that were stored for a period of time in DMSO and compounds that were reconstituted just before conducting the assay. When only one value is listed, it is the inhibition value of the compound originally received from the NINDS (no commercial compound available).
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoxsuprine Hydrochloride</td>
<td><img src="image1" alt="Structure" /></td>
</tr>
<tr>
<td>Lysyl-tyrosyl-lysine Acetate</td>
<td><img src="image2" alt="Structure" /></td>
</tr>
<tr>
<td>Cefoperazone Sodium</td>
<td><img src="image3" alt="Structure" /></td>
</tr>
<tr>
<td>Tyrothricin</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
</tbody>
</table>

**Figure 2.** Structures containing phenol hydroxyl groups.
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methacycline</td>
<td><img src="image" alt="Methacycline" /></td>
</tr>
<tr>
<td>Meclocycline sulfosalicylate</td>
<td><img src="image" alt="Meclocycline sulfosalicylate" /></td>
</tr>
</tbody>
</table>

**Figure 3.** Structural similarity between methacycline and mecloycline sulfosalicylate.
Figure 4. Structural comparison of quinalizarin and naringin.
<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Structure</th>
<th>IC₅₀ and EC₅₀ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoporphyrin IX</td>
<td><img src="image1" alt="Structure" /></td>
<td>Wetzel: 12 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neri: 0.005 µM</td>
</tr>
<tr>
<td>Cephalosporin C Sodium</td>
<td><img src="image2" alt="Structure" /></td>
<td>Wetzel: 79 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neri: 2.000 µM</td>
</tr>
<tr>
<td>Quinalizarin</td>
<td><img src="image3" alt="Structure" /></td>
<td>Wetzel: 91 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neri: 2.800 µM</td>
</tr>
<tr>
<td>Perphenazine</td>
<td><img src="image4" alt="Structure" /></td>
<td>Wetzel: 125 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neri: 1.000 µM</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td><img src="image5" alt="Structure" /></td>
<td>Wetzel: 133 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neri: 1.800 µM</td>
</tr>
<tr>
<td>Oxolinic Acid</td>
<td><img src="image6" alt="Structure" /></td>
<td>Wetzel: 321 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neri: 2.300 µM</td>
</tr>
<tr>
<td>Compound Name</td>
<td>Structure</td>
<td><strong>IC₅₀ and EC₅₀ values</strong></td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>-------------------------</td>
</tr>
</tbody>
</table>
| Ebselen       | ![image](https://example.com/image1.png) | Wetzel: 408 μM  
Neri: 0.200 μM |
| Cannabidiol   | ![image](https://example.com/image2.png) | Wetzel: 743 μM  
Neri: 0.020 μM |
| Naringin      | ![image](https://example.com/image3.png) | Wetzel: 7.8 mM  
Neri: 0.800 μM |
| MCI-186       | ![image](https://example.com/image4.png) | Wetzel: IC₅₀ 252 mM  
Neri: EC₅₀ 0.016 μM |
| Celastrol     | ![image](https://example.com/image5.png) | Wetzel: No effect  
Neri: 0.400 μM |
| Glibenclamide | ![image](https://example.com/image6.png) | Wetzel: No effect  
Neri: 2.300 μM |
| Terfenadine   | ![image](https://example.com/image7.png) | Wetzel: No effect  
Neri: 0.900 μM |

**Figure 5.** Structures for the 13 most active compounds with *C. elegans*, and comparison of IC₅₀ values found in the Wetzel group's microplate assay with EC₅₀ values found in Dr. Neri's work with *C. elegans.*
Figure 6. Structural similarity of nortriptyline, perphenazine, and oxolinic acid to hycanthone.
Figure 7. Linear regression graph of the first 8 compounds from Table 2 (protoporphyrin IX to cannabidiol) to show relationship between EC<sub>50</sub> values in C. elegans testing (x-axis) and IC<sub>50</sub> values in the microtiter plate assay (y-axis).