Identification of Potential Therapeutic Drugs for Huntington’s Disease using Caenorhabditis elegans

Cindy Voisine1,4,*, Hemant Varma2,*, Nicola Walker1,4,*, Emily A. Bates1,4, Brent R. Stockwell2,3, Anne C. Hart1,4

1 Massachusetts General Hospital Cancer Center, Charlestown, Massachusetts, United States of America, 2 Department of Biological Sciences, Columbia University, Fairchild Center, New York, New York, United States of America, 3 Department of Chemistry, Columbia University, Fairchild Center, New York, New York, United States of America, 4 Harvard Medical School Department of Pathology, Boston, Massachusetts, United States of America

INTRODUCTION

Drug discovery for late onset neurodegenerative diseases is a major challenge. In large part, the complexity of treating these disorders results from our insufficient understanding of the contributions of multiple pathways on disease pathophysiology. Furthermore, since the pathology of these disorders is often only discernable in aged populations, testing the therapeutic value of small molecules in vertebrate disease models requires time consuming and costly experimental designs. The development of rapid and inexpensive in vivo assays to evaluate the numerous candidate compounds identified in high-throughput screens is therefore of paramount importance.

Invertebrate model organisms such as C. elegans provide an attractive alternative for prioritizing lead compounds in the early stages of drug development for age-related diseases [1–3]. C. elegans has several characteristics that make it ideal for drug testing— including a short lifecycle, small size and the ease of culturing in liquid. Furthermore, decades of neurobiological and antiparasitic drug studies in C. elegans provide a strong foundation for use of this organism in therapeutic compound identification [1–3].

Huntington’s Disease (HD) is caused by expansion of a polyglutamine (polyQ) tract in the huntingtin protein leading to neurodegeneration that is age and polyQ tract length dependent [4]. In this study, we use a C. elegans model of polyQ neurotoxicity in which the N-terminal 171 amino acid fragment of human huntingtin protein containing an expanded polyglutamine tract (150Qs) is expressed in neurons. Degeneration and cell death in this model is dependent on both age and polyglutamine tract length, recapitulating these aspects of the human disease [4–6].

We tested a collection of compounds that have been previously described to decrease degeneration in cell culture/animal models of polyQ toxicity for their ability to protect C. elegans neurons from the toxic effects of an expanded huntingtin polyglutamine fragment. We developed, optimized and validated new assays for use in rapid assessment of drug efficacy using C. elegans HD models.

Of the compounds tested, we found that two FDA approved drugs, mithramycin (MTR) and lithium chloride (LiCl), reduced polyQ toxicity in the C. elegans model.

An important determinant of neurodegenerative diseases is the aging process. However, the mechanistic links between aging and the cellular pathways leading to neurodegeneration are not well understood. The forkhead transcription factor DAF-16, which mediates the effects of the insulin-like signaling pathway on aging, has been shown to play a role in polyQ aggregation. Mutations that reduce insulin signaling, derepress DAF-16 leading to an increase in lifespan and stress resistance [7,8], whereas RNAi based knockdown of daf-16 accelerates polyQ aggregation and toxicity [9] suggesting that DAF-16 transcriptional targets not only promote longevity but also prevent polyQ aggregation. Despite the

Background. The prolonged time course of Huntington’s disease (HD) neurodegeneration increases both the time and cost of testing potential therapeutic compounds in mammalian models. An alternative is to initially assess the efficacy of compounds in invertebrate models, reducing time of testing from months to days. Methodology/Principal Findings. We screened candidate therapeutic compounds that were identified previously in cell culture/animal studies in a C. elegans HD model and found that two FDA approved drugs, lithium chloride and mithramycin, independently and in combination suppressed HD neurotoxicity. Aging is a critical contributor to late onset neurodegenerative diseases. Using a genetic strategy and a novel assay, we demonstrate that lithium chloride and mithramycin remain neuroprotective independent of activity of the forkhead transcription factor DAF-16, which mediates the effects of the insulin-like signaling pathway on aging. Conclusions/Significance. These results suggest that pathways involved in polyglutamine-induced degeneration are distinct from specific aging pathways. The assays presented here will be useful for rapid and inexpensive testing of other potential HD drugs and elucidating pathways of drug action. Additionally, the neuroprotection conferred by lithium chloride and mithramycin suggests that these drugs may be useful for polyglutamine disease therapy.
pivotal role that growth and aging play in neurodegenerative disease, we found that LiCl and MTR protect C. elegans neurons in the absence of growth and through a daf-16 independent pathway suggesting that these compounds may target pathways that are specific to neurodegeneration. Thus, the integration of pharmacological and genetic examination of drugs, in C. elegans HD models that we describe, should accelerate the identification of interventions for HD along with insight into mechanism of drug action.

RESULTS

Compound concentration range for screening in C. elegans: Food Clearance Assay

We tested a collection of compounds that have demonstrated therapeutic value in either cell culture and/or animal models of polyQ toxicity for screening in our C. elegans HD models (Table 1). These candidates represent compounds that may protect against polyQ toxicity by affecting a variety of cellular pathways. To efficiently evaluate the effects of these candidates on neurodegeneration and neuronal cell death, we first established a systematic method for selecting optimal drug concentrations to assess in our C. elegans HD models (see Methods). Compounds were tested in a dose dilution series in the food clearance assay (Figure 1 and Figure S1) starting at the highest soluble concentration. Taking advantage of the short life cycle and the ability of C. elegans to grow in liquid culture of E. coli, we evaluated compounds by monitoring the rate at which the E. coli suspension (food source) was consumed. Each adult is capable of producing hundreds of progeny that rapidly consume the limited E. coli supply. As a result, the OD of wells without compound drastically decreases in 3 days. Any drug that decreases C. elegans growth, survival or fecundity would result in a dose dependent reduction of the rate at which food is cleared (consumed) in a well. For example, addition of 5 mM LiCl to the culture showed no effect on food clearance compared to control animals, whereas animals exposed to 10 mM LiCl had delayed food clearance (Figure 1B). Visual inspection confirmed that animals treated with 10 mM and 25 mM LiCl were smaller in size compared to untreated animals whereas animals treated with 5 mM LiCl were unaffected (Figure 1C and data not shown). Furthermore, animals exposed to 50 mM and 100 mM LiCl did not produce progeny over the time course of the experiment (data not shown), which correlated with the lack of clearance of the E. coli food source. For all compounds, optimal concentrations for C. elegans were similarly assessed using the food clearance assay (Table 1). This simple assay for determining a compound’s concentration range to test in C. elegans uses a small amount of compound and is amenable to high-throughput format, making it applicable to any C. elegans drug study.

Screening neuroprotective compounds in C. elegans polyQ model

In order to test compounds in an efficient manner, we decided to reduce the amount of time required to test drug efficacy. Relatively long assay duration may also be unsuitable for testing compounds that are unstable under assay conditions. We utilized a genetic mutant background (pqe-1 loss of function) that accelerates polyQ mediated neurodegeneration and cell death in C. elegans from 7 days to 2–3 days. We evaluated neuronal death by monitoring loss of expression of a GFP reporter construct in the bilateral ASH sensory neurons in pqe-1;Htn-Q150 animals, a genetically enhanced model of polyQ toxicity [10] (Figure S2). In pqe-1;Htn-Q150 animals, the vast majority (>90%) of ASH neurons undergo cell death in less than three days (Figure S2).

Synchronized pqe-1;Htn-Q150 L1 larvae were treated with and without drug for three days, then GFP expression was monitored to assess ASH neuronal viability (Figure 2A). Of the candidates tested, we identified three protective compounds, mithramycin (MTR), trichostatin A (TSA) and lithium chloride (LiCl) (Table 1). All three compounds caused a dose-dependent neuroprotection in

### Table 1. Compounds screened in C. elegans polyQ model for neuroprotection.

| Drugs tested | Putative Mechanism | TC 50 Food clearance (mM) | Conc. Tested (mM) | EC 50 (mM) | Rescue in C. elegans | Activity reported in HD model(s) |
|--------------|--------------------|--------------------------|-------------------|-----------|----------------------|---------------------------------|
| LiCl         | GSK-3β inhibitor   | 25                       | 10–100            | 25        | Yes                  | Cell culture [31] D. melanogaster [32] |
| Mithramycin  | DNA binding        | 0.003                    | 0.01–1            | 0.5       | Yes                  | M. musculus [33] |
| Trichostatin A (TSA) | HDAC inhibitor | 1                        | 0.16–1           | 0.16      | Yes                  | Cell Culture [34] |
| SAHA*        | HDAC inhibitor     | 2                        | 2                 | n.a.      | Yes                  | M. musculus [36] |
| Taxol        | Cytoskeletal dynamics | none                    | 0.4               | n.a.      | no effect            | Cell culture [38] |
| Congo Red    | Aggregation inhibitor | 0.5                   | 0.12–0.5         | n.a.      | no effect            | M. musculus [39] |
| Cystamine    | Transglutaminase inhibitor | 300                  | 150               | n.a.      | no effect            | M. musculus [40–42] |
| Budesonide   | Glucocorticoid receptor agonist | none               | 0.8               | n.a.      | no effect            | D. melanogaster [43] |
| Paraquat     | Free radical generator | 3                      | 3                 | n.a.      | no effect            | Cell culture [45] |

All compounds were tested in pqe-1 enhanced background in the presence of food, E. coli.

n.a. = Not applicable as these compounds were not active.

*SAHA was only tested and found to be active at a single concentration (2 mM). Therefore, an EC50 was not determined.

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Figure 1. Concentration of compounds for testing was assessed using a food clearance assay. (A) Flow diagram of the food clearance assay. 20 newly hatched L1 animals were incubated at 25°C in E. coli at a final OD (A_{595}) of 0.6 in 96 well microtiter plate wells containing varying drug concentrations. The OD of the microtiter plate was measured daily for 5 days. (B) The OD of E. coli is reported daily for each concentration of LiCl. The mean OD is calculated for each day from triplicate samples and plotted over time. Error bars represent SEM. Food clearance assays were also performed on trichostatin A and mithramycin, (Figure S1). (C) Animals treated with no drug and indicated LiCl concentrations. Animals treated with 50 mM or 100 mM LiCl are alive but concentrations above 100 mM LiCl cause death (data not shown). Scale bar is approximately 75 μm.

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the pqe-1 sensitized background (Figure 2B). MTR and TSA affect transcriptional activity [11,12] and LiCl is a drug used clinically to treat individuals with bipolar disorders and is a potential inhibitor of GSK3β [13]. Previous studies in C. elegans demonstrated that TSA reduced polyQ mediated neurodegeneration [14], validating this approach. Htn-Q150 transgene expression was not altered by drug treatment based on RT-PCR (Figure S3). Based on these results, we conclude that a genetically sensitized background (eg. pqe-1) can be used to dramatically reduce the amount of time required to accurately assess effects of drugs on neuronal cell death.

Neuroprotection in an age-dependent C. elegans polyQ model
To determine if neuroprotection was dependent on pqe-1, we tested candidates in the Htn-Q150 only background that exhibits age-dependent neurodegeneration but no cell death. Late onset degeneration of ASH sensory neurons was monitored by defects in dye uptake, a previously described assay [6] (Figure S2).

One limitation due to the prolonged duration (7 days) of the aging assay is the production of progeny (200–300 per adult) by the aging animals. Since the progeny grow to adulthood in 3 days, they are difficult to distinguish from the aged parent by day 7. A novel genetic strategy was employed to prevent progeny development. We crossed Htn-Q150 into a genetic background (pha-1) in which progeny fail to feed and grow at the restrictive temperature of 25°C [15]. Introducing pha-1(e2123) to eliminate progeny development permits the use of liquid cultures and facilitates any C. elegans aging study (see Methods).

To assay compound efficacy, synchronized pha-1;Htn-Q150 L1 larvae were treated with and without drug for seven days, then scored for degeneration using the dye-filling assay (Figure S2). Introducing the pha-1 mutation had no effect on the dye-filling of ASH neurons or the polyQ dependent neurodegeneration (data not shown and Figure S2). LiCl decreased polyQ neurodegeneration in a dose dependent manner (Figure 3B) while equivalent concentrations of sodium chloride (NaCl) had no effect (data not shown), showing the specificity of LiCl's effect. Consistent with previous findings [14], the HDAC inhibitor trichostatin A (TSA) also suppressed neurodegeneration in the aging assay format (Figure S4). The confirmation of compound activity in the age dependent Htn-Q150 model validated the utilization of the pqe-1 sensitized strain as a rapid means of identifying HD therapeutic compounds.

Dissociating neuroprotection from growth, development and aging
Since the aging process is a critical but poorly understood contributor to late onset neurodegenerative diseases [9,16,17], compounds that affect aging may indirectly affect degeneration. To distinguish drug effects on neuronal cell death from those on growth, development and aging rates, we applied two strategies: (i) we modified our assay format to prevent growth of animals for the duration of the assay (ii) we genetically altered the well characterized insulin signaling pathway that affects aging. To modify our assay, we took advantage of the growth arrest induced by starvation of L1 stage C. elegans larvae. When embryos hatch in the absence of food, their development is arrested until food is available [18,19]. Since starved L1 animals do not grow, the effects of compounds on neuronal cell death can be evaluated independent of their effects on growth and development. A further advantage is that drugs can be tested in the absence of bacteria (E. coli food source) that could degrade or metabolize compounds under analysis. Using a starvation assay format (Figure 4A), we found that ASH neurons continue to undergo neuronal cell death in pqe-1;Htn-Q150 animals that are growth arrested by the absence of food (data not shown). This finding suggests that certain pathways that affect polyQ neurodegeneration are distinct from pathways that effect growth and development.

We tested LiCl and MTR in this starvation assay format. Both LiCl and MTR protected ASH neurons of starved animals in a dose-dependent manner (Figure 4B). Equivalent concentrations
of NaCl as that of LiCl had no effect on neuronal cell death demonstrating specificity of the compound’s effect (data not shown). Using this assay modification, higher concentrations of compounds that interfere with *C. elegans* development and growth were tested in the starvation assay (**Figure 1C** and **Figure 4B**). Therefore, testing drugs in the L1 arrest assay can distinguish contributions of specific drugs on organismal development and aging, providing a clearer demonstration of the neuroprotective properties of compounds.

The insulin signaling pathway, a critical regulator of longevity in *C. elegans*, is also a modifier of polyQ toxicity. RNAi mediated knockdown of *daf-16* decreases lifespan and leads to early onset of polyQ aggregation [9]. To determine if the neuroprotective effects of LiCl and MTR are dependent on components of the insulin signaling pathway, we crossed a *daf-16* null mutation into *pqe-1;Htn-Q150* animals and tested the effects of LiCl and MTR on neuronal cell death in the starvation assay. We found that both compounds remained neuroprotective in the *daf-16* mutant animals in a dose dependent manner (**Figure 4C**), suggesting that LiCl and MTR suppress polyQ mediated neuronal cell death independent of DAF-16 activity. The dissociation of the neuroprotective effects of compounds from growth and specific aging pathways allows a mechanistic classification of therapeutic compounds.

**Combinatorial effects of LiCl and MTR**

A number of therapies for complex diseases use combinations of drugs that allow targeting of multiple mechanisms and allow therapeutic efficacy at lower compound concentrations, limiting...
toxicity of single agents. We also investigated the utility of our C. elegans assay in drug combinatorial studies by testing LiCl in combination with MTR on C. elegans polyQ toxicity in the starvation assay. Combining 25 mM LiCl and 0.5 mM mithramycin in the starvation assay format increased ASH neuron survival compared to the protection afforded by either compound alone (Figure 4D).

**DISCUSSION**

In this study, we have devised strategies for efficiently assessing therapeutic efficacy of compounds in C. elegans HD models (Figure 5). Our methods exploit the strengths of C. elegans model for drug discovery, including convenient and precise visualization of live neurons, small-scale liquid cultures that significantly reduce the amount of drug required for testing as well as genetic approaches to implicate specific cellular pathways in a compound’s mechanism of action. These assays could be utilized for prioritizing the large numbers of hits already identified by high-throughput screens for HD and other polyQ diseases [20–23].

The normal aging process is intricately linked to late onset neurodegenerative diseases. However, the influence of aging on cellular events that impact neurodegeneration is unclear. To dissect the interplay of pathways involved in neurodegeneration from those involved in aging, we combined genetic manipulations with a novel assay that utilizes growth-arrested animals. Our studies demonstrate that LiCl and MTR exert their neuroprotection in a daf-16 independent manner. However, starvation of animals may activate additional stress responsive transcription factors such as heat shock factor (HSF1). HSF1 regulates the heat shock response and also influences longevity [17,24,25]. Furthermore, HSF1 RNAi enhances polyQ aggregation [26] raising the possibility that HSF1 or other stress activated pathways may function as molecular targets of these compounds. However, the fact that neurons continue to die under conditions of starvation suggests that the stress responses induced by starvation are insufficient to abrogate the neurodegenerative process. Thus, the combination of strategies outlined in the study allows the separation of drug effects on neurodegeneration from those on growth and stress related aging pathways.

Though we focus on neurodegeneration in HD models, similar strategies may apply to other age dependent phenotypic readouts. For example, deletion of the C. elegans dystrophin gene, the homolog of a gene involved in Duchenne’s Muscular Dystrophy, in combination with a second mutation in the myogenic factor MyoD leads to progressive muscle degeneration [27,28]. Using the strategies described in this study, the protective effect of
Figure 5. Schematic Diagram of Compound Testing Strategy. (1) A range of concentrations for each compound for testing in C. elegans was established using the food clearance assay. (2) The protective effects of compounds on polyglutamine neurodegeneration and cell death were assessed in pqe-1(Htn-Q150) animals treated with compounds for 3 days. (3) To determine if neuroprotective effects of compounds were dependent on pqe-1, compounds were retested in animals expressing Htn-Q150 for 7 days. (4) To distinguish drug effects on neuronal cell death versus effects on growth and/or development, synchronized pqe-1/Htn-Q150 L1 animals were incubated in the presence of drugs without food for 2 days. (5) The neuroprotective effects of compounds on the aging process can be tested by introducing mutations (daf-16) in components of specific aging-related (insulin signaling) pathways.

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compounds on muscle degeneration could be conducted rapidly. These C. elegans disease models should be valuable tools for rapid identification of potential therapeutic compounds for aging related human diseases and cellular pathway elucidation of any compounds that are active in these models.

MATERIALS AND METHODS

Strains
C. elegans N2[wild-type] and GE24[pha-1(e2123)], E. coli food sources OP50 and HB101 (OP50 for compound efficacy and aging assays and HB101 for sensitized and food clearance assays) were obtained from the Caenorhabditis elegans Genetics Center. Htn-Q150[rtIs11] expressing strains with and without pqe-1[rtIs11] were previously described [6,10], daf-16[mgDf47] was crossed into pqe-1[rtIs11] mutant strain expressing Htn-Q150[rtIs11] marked with dpy-11[rtIs224]. All mutations were homozygous in the final strain for analysis. All strains were cultivated on nematode growth media (NGM) plates at 15°C [29].

Food Preparation
E. coli were grown overnight at 37°C in Luria broth [LB] media, pelleted by centrifugation, frozen at –70°C, and then resuspended at a final OD of 0.5 (595 nm) for the aging assay or an OD of 6.6 in nematode S-medium [29] (supplemented with 100× streptomycin/penicillin [Invitrogen, #15140-122] and nystatin [Sigma, #N1638]) for all other food based assays. Note the lower OD (~0.6) in plates was due to a decreased path length of the 60 µl final suspension, in a well, compared to a 1 cm path length in a spectrophotometer.

Compound Preparation
All compounds were purchased from Sigma (St. Louis, MO). Stock solution of 1 M lithium chloride (LiCl) in water, 10 mM mithramycin (MTR) in PBS and 5 mg/ml trichostatin A (TSA) in DMSO were stored at room temperature (LiCl), 10–14 days at 4°C (MTR) and at –20°C (TSA). Compounds were diluted into E. coli suspension or S-medium (starvation assay) to the desired concentrations. 1 ml of the final mixture was added per well of a 24-well polystyrene plate for the aging assay. For the remaining assays, 50 µl of the final mixture was added per well in a 96-well polystyrene plate.

C. elegans Preparation
We used newly hatched C. elegans, collected as L1 larvae at 15°C. Specifically, adults were lysed, fertilized eggs were collected by hypochlorite treatment of gravid adults and suspended in S-medium for 24–30 hours at 15°C and L1 larvae were allowed to hatch overnight in the absence of food [29]. pha-1(e2123) is a temperature sensitive loss of function allele that affects normal pharyngeal development [15]. A normal pharynx forms at the permissive temperature of 15°C in newly hatched L1 animals while the pharynx fails to develop in pha-1(e2123) animals hatched at 25°C. C. elegans L1 animals do not initiate growth in the absence of food, resulting in a synchronous population of arrested L1 animals. Introduction of a food source (E. coli) results in growth; animals become adult hermaphrodites within 48 hours at 25°C. This L1 growth arrest and recovery provides an efficient means of collecting large numbers of synchronized animals for assays.

Food Clearance Assay
The effect of compounds on C. elegans physiology is monitored by the rate at which the E. coli food suspension was consumed, as a read out for C. elegans growth, survival or fecundity. Approximately 20–30 L1 synchronized animals per 10 µl of S-media were added to an E. coli suspension. Microtiter plates containing animals and drugs were incubated at 25°C. The absorbance (OD 595nm) was measured daily using a Vmax Kinetic microplate reader (Molecular Devices). We used N2 animals when determining compound concentration. Introduction of pha-1(e2123), pqe-1[rtIs13], daf-16[mgDf47] or Htn-Q150[rtIs11] had no effect on overall growth or health of the animal compared to N2 in the presence of drug at assay temperatures.

Scoring Degeneration and Cell Death
At time points indicated, animals were collected by centrifugation, washed in S-media and immobilized with 5 mM sodium azide on a 2% agarose pad. GFP fluorescence was scored using an Axiosplan2 fluorescence microscope (n = 100 ASH neurons). For the aging assay, degeneration was assessed by dye-filling [6]. Animals were incubated for 2 hours in 1,1’dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine perchlorate (DiD) (Molecular Probes) and immobilized as above for scoring ASH dye filling (n = 50 ASH neurons). To determine statistical significance, we compared the results from parallel experiments using 2x2 contingency tables comparing the number of GFP+ and GFP- neurons in control vs. specific concentrations of a compound. We determined the statistical significance using a Fisher’s exact test for each of the three independent experiments and reported the
The mean OD is calculated for each day from triplicate samples reported daily for each concentration of TSA (A) and MTR (B).

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