The Identification of Chemical Compounds that Decrease Cellular Levels of Toxic Huntington's Disease Protein Through a Novel Cell-Based Assay

by

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ABSTRACT

Huntington's disease (HD) is a progressive degenerative neurological disorder. Individuals who inherit the IT15 gene with an expansion of the CAG repeat region inevitably succumb to increasingly sever motor, psychological, and cognitive symptoms. I sought to develop an assay system with the capability for identification of chemical compounds that selectively decrease the intracellular levels of disease-causing expanded polyglutamine huntingtin (Htt) protein without reducing the intracellular levels of the potentially protective normal Htt. To achieve this goal I designed a cell-based assay using the enzymatic activity of E. coli β-galactosidase as a reporter for Htt protein levels. I expressed either expanded (97Q) or normal (23Q) Htt fused to the β-galactosidase alpha-subunit (α) in an inducible fashion in PC12 cells which also expressed the β-galactosidase delta-subunit (Δ). Complementation between these expressed subunits allowed the formation of functional β-galactosidase. The level of β-galactosidase activity in these Δ-α 97Q and Δ-α 23Q cells directly correlated with the amount of α 97Q and α 23Q fusion protein levels, indicating that β-galactosidase activity could be used as a reporter in this system for Htt protein levels. I implemented this cell-based assay as a secondary assay to characterize a group of compounds that had been initially identified in a High Throughput Screen because they reduced levels of expanded Htt-fragment fused to GFP. Of the 34 compounds characterized in the β-galactosidase Δ-α 97Q assay, dose response curves and counter-screening with Δ-α 23Q cells revealed that seven compounds decrease β-galactosidase activity only in Δ-α 97Q cells. Immunofluorescence demonstrated that two compounds decrease levels of expanded but not normal Htt proteins in the cells. Finally, tests of toxicity on Htt^{Q103} PC12 cell lines, which show specific toxicity following expression of expanded Htt, revealed a significant correlation with the results from the β-galactosidase assay and the identification of at least one compound which continued to meet the criteria for therapeutic intervention in HD. These results support the feasibility of the development of an HD therapeutic strategy based on small molecules which cause a specific reduction of intracellular expanded Htt protein levels and suggest a program of development for such molecules.

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CHAPTER I

HUNTINGTON'S DISEASE AND THE SEARCH FOR A TREATMENT

In 1872, George Huntington first published a description of a hereditary form of chorea [1], the disease which now bears his name. He noted three hallmarks of Huntington's disease (HD), aside from chorea: 1. Psychological symptoms: high likelihood of suicide, insanity, and socially unacceptable behavior; 2. Dominant inheritance: affected individuals have an afflicted parent; the lineage of children from an unaffected parent who do not manifest the disease also never exhibit symptoms; 3. Late-onset: patients typically begin showing symptoms at 30-40 years of age.

Even though Huntington did not think that these observations were "of any great practical importance", and presented the information "merely as a medical curiosity", HD is now a recognized disease affecting 5-10 out of 100,000 people. The tremendous effort put forth by the scientific community, particularly since the discovery of the gene responsible for the disease in 1993, has lead to a detailed understanding of the disease's cellular mechanisms. This knowledge has in turn led to significant therapeutic promise. However, there is still no treatment for this devastating disease.

HD SYMPTOMS IN MOVEMENT, PSYCHOLOGY, AND COGNITION

HD is now characterized by the progressive degeneration in motor, psychological, and cognitive abilities [2]. Typically, chorea begins in the hands and feet, expanding over the years to the limbs and face, while increasing in intensity. The symptoms include inability to control the tongue, eyelids, and eye movements. Many HD patients exhibit aggressiveness, apathy, and depression. HD patients are five times more likely to commit suicide than the general population. The decline in cognitive ability affects memory, attention, concentration, emotional processing, spatial manipulation, and eventually dementia.

HD IS A DOMINANTLY INHERITED DISEASE

The genetic cause for HD is an expansion of the CAG repeats in the first exon of the HUNTINGTINgene, located on chromosome 4 [3, 4]. This expansion in CAG repeats translates into an expanded poly-glutamine (polyQ) region in the huntingtin (Htt) protein. Unaffected individuals have Htt with 6-35Q (normal Htt), while HD patients have Htt with 40-100Q (expanded Htt). The variation in the polyQ expansion in patients correlates to age of onset, where patients with a longer polyQ region have a tendency to present with symptoms earlier in life [5]. However, there is no correlation between the length of the polyQ region and the rate of disease progression.

HD ANIMAL MODELS

While physical symptoms are easily described in HD patients, the underlying causes of the symptoms can not be studied in humans. In order to more closely study the disease mechanism, many HD animal models have been created by expressing expanded Htt. No animal model is a perfect recreation of the disease in humans, but each one does recapitulate some of the motor, psychological, and cognitive dysfunctions. Furthermore, some of these animal models are widely used to determine efficacy of potential drug therapies for HD.

Drosophila

Neurodegeneration [6-8], motor dysfunction, and premature death [2] are HD phenotypes that have been recapitulated in *Drosophila* models. The abnormalities observed in ommatidia, clusters of eight photoreceptor neurons that make up the *Drosophila* eye, serve as evidence of neurodegeneration. Motor function can easily by assayed by observing the larva's path through agar and the adult's flying or walking abilities. Finally, the three month life-span of normal adult *Drosophila* provides a relatively quick way to determine premature death.

The N-terminal Htt *Drosophila* model expresses specifically in the eye the first 170 aa of Htt with either 75Q or 120Q [9]. Up through the pupal stage, all animals have normal eyes, indicating that expanded Htt expression does not affect the development of the ommatidia. However, degeneration of the ommatidia is observed in Htt-120Q and Htt-75Q containing animals, with onset at adulthood day 10 and 30, respectively. Throughout early development,

the expression of Htt is cytoplasmic in both *Drosophila* lines. In the transition from pupa to adulthood, the expression of Htt-120Q begins to shift towards the nucleus. By day 10, this expression becomes punctated.

Another HD *Drosophila* model expresses the first 548 aa of Htt with 128Q [10]. When the transgene is expressed specifically in the eye, the ommatidia degenerate during adulthood. When the transgene is expressed in all neurons, abnormal motor functions are observed in larvae and adults. This expression of Htt-128Q results in premature death. In this *Drosophila* model, cytoplasmic but not nuclear aggregated Htt is found in the late larval stage.

These *Drosophila* models are beneficial for determining the efficacy of a potential HD drug treatment for several reasons. First, the effects of a drug on the extent and timing of ommatidia degeneration can easily be quantified, which could parallel the drug's effect on neurodegeneration seen in humans [6-8]. Second, a delay or prevention of lethality is another measure of a drug's efficacy that can easily be assessed, and potentially beneficial to humans. Third, the short life-span and large number of progeny of *Drosophila* allows for statistically significant data from such drug tests.

Mouse

Many aspects of HD have been recapitulated in mouse models, although no one model shows all. The neurodegeneration observed in humans [6-8] can be studied in mice by measuring whole-brain weight, or by counting nuclei. Motor dysfunction [2] can be studied by multiple protocols. One popular protocol is the accelerating rotarod, where a mouse is placed on a rotating rod and the latency to fall is noted. Other protocols include gait analysis and quantification of limb clasping. Cognitive ability [2] can be assayed by protocols that require learning and memory. One such protocol is the Morris water maze, where the mice must learn and remember the location of a hidden platform in a pool of water. Another learning protocol is the T-maze, where the mice must learn which arm of a T-shaped maze contains the food. The weight loss observed in HD patients [11] can easily be measured in mice by noting daily body weights. Finally, premature death can be measured.

R6/2 and R6/1 mice

The R6/2 and R6/1 mice were the first HD mouse models [12]. They express exon one of Htt and an expanded polyQ, where R6/2 mice have 150Q and R6/1 mice have 115Q. At 3-4 weeks of age, the R6/2 mice show dysfunction in cognitive abilities as assayed by the Morris water maze and T-maze [13]. At eight weeks of age, motor dysfunction is visible in R6/2 mice by rotarod, limb clasping, and gait abnormalities, whereas the onset for R6/1 mice is around 13 weeks of age [12]. R6/2 mice start to lose weight around eight weeks of age. R6/2 mice tend to die between 13-15 weeks of age, while R6/1 mice live longer than one year. There is significant loss in brain weight in 12 week old R6/2 mice and 18 week R6/1 mice [14], although this is due to cell atrophy and not cell loss [15, 16]. Striatal and cortical aggregates are first visible in 3-4 week old R6/2 and eight week old R6/1 mice [17, 18].

The R6/2 mice are the most commonly used mouse model for testing the efficacy of potential drugs for HD treatment, discussed in the next section. One reason for this is that because of the early onset of symptoms, the drug trials are quick. This allows for a larger number of animals in the drug trials resulting in good statistical analysis of data.

N171-82Q mice

The N171-82Q mice express the first 171 aa of Htt with 82Q in neurons [19]. The first HD-related symptom to manifest is weight loss at two months of age. The motor dysfunctions manifest in rotarod, limb clasping, and gait abnormalities begin at three months. Finally, these mice die at 5-6 or 8-11 months, depending on the line. The expanded Htt protein is found diffusely in the nucleus of neurons, but also in aggregated form in the cortex and striatum. Neurodegeneration is evident in the striatum.

The N171-82Q mice have been used to test several drug treatments for HD, discussed in the next section. Although the onset of symptoms is later than in the R6/2 mice, the onset is a relatively quick 2-3 months. This later onset and prolonged disease progression can be an advantage because the disease mechanism being targeted might require a prolonged exposure to a drug in order to see an effect.

Full-length Htt mice

Two full-length expanded Htt mouse models, cDNA with 89Q [20] and YAC with 72Q [21], have a similar time-line in the onset of symptoms. Although a small percentage of striatal neurons have nuclear aggregates, there is significant cell loss in the striatum at 12 months of age. Another full-length Htt mouse model with 128Q, YAC128, begins to fail at the rotarod test at six months of age, and neuronal cell loss is observed at 12 months of age [22]. None of these models are ideal for drug testing because of the late-onset of motor symptoms and neuropathology. However, these mouse models should be used for potential drugs that target full-length Htt, since R6/2 and N171-82Q mice only express N-terminal fragments of Htt.

Knock-in polyQ expansion of mouse Htt

The mouse homolog of the HD gene, Hdh, is 91% identical to the human gene, with exon one having 100% homology [23, 24]. In order to examine the effects of expanded Htt with a normal expression pattern and protein levels, multiple groups have inserted expanded polyQ tracts into the endogenous mouse Hdh gene [25-34]. Knock-in mice with 150Q, 140Q and 109Q have abnormal gait beginning at 12 months, 12 months, and 24 months, respectively [25-27]. The Hdh-150Q mice also exhibit limb clasping and rotarod dysfunctions by 12 months of age [27]. Although none of the knock-in mice show neuronal loss, those with 72-80Q and 140Q do have axonal atrophy at 17-22 months and 14 months of age, respectively [32, 33]. Aggregates are visible only in the striatum of Hdh-94Q mice [34], whereas they are more widespread throughout the brain in knock-in mice with 109Q, 140Q, or 150Q [26-28]. The differences between the onset of symptoms in the different knock-in mouse lines are partially due to the length of the polyQ tract. In addition, genetic modifiers in the different mouse strains used to insert the polyQ tract can be another factor. Similar to the transgenic full-length mouse models, these knock-in mice could be used to test drugs that target the full length Htt protein.

DYSFUNCTIONAL CELLULAR PROCESSES IN HD

Htt is widespread throughout the body, with the highest expression in the central nervous system [35, 36]. In cellular and animal models of HD, the majority of dysfunctional processes are attributed to a gain of function by expanded Htt, although loss of function of normal Htt also

contributes. The levels of normal Htt are decreased in HD patients since they express normal Htt from only one allele. The 50% reduction in normal Htt levels is not the sole cause of HD symptoms since people with a deletion in chromosome 4 that includes the HUNTINGTINgene do not exhibit symptoms of HD [37]. However, the levels of normal Htt in HD patients are even further reduced due to sequestration by expanded Htt.

Greater understanding of the dysfunctional cellular processes in HD has led to the identification of potential drug therapies, described below. However, to date, success in treating HD has been minimal [38]. An underlying reason for this limited success may be that each drug tested thus far has been directed towards a single cause of cellular dysfunction in HD, while as I will discuss below, there are in fact multiple pathways which have been shown to contribute significantly to cellular dysfunction in HD. So, even if a drug were to be maximally effective in addressing a single cause of cellular dysfunction, additional pathways to cellular dysfunction may remain active during treatment with such a drug, resulting in the continued presence and progression of disease symptoms.

Increased cell death

Brains from HD patients are significantly smaller due to dramatic cell loss in the striatum and partial loss in the cortex [6, 7]. MRI scans of individuals who were an average 22 years away from the predicted age of onset show that the striatum is already smaller [8]. Cell death in HD has been attributed to the specific mechanisms of excitotoxicity and apoptosis, along with death due to general cellular dysfunction.

Excitotoxicity

Excitotoxicity refers to cell death caused by continuous excitatory stimulation of a neuron, such as that caused by the binding of a high affinity agonist to glutamate-receptors causing intracellular calcium concentrations to rise to toxic levels. Normal Htt directly binds to the scaffold protein PostSynaptic Density-95 (PSD-95), and stabilizes it at the synapse while expanded Htt has a lower affinity to PSD-95 resulting in a higher amount of PSD-95 available to stimulate NMDA receptors [39]. Over-stimulated NMDA receptors have been reported in cell lines expressing expanded but not normal Htt [40, 41].

Inhibitors of the NMDA pathway can decrease the amount of calcium influx and have had some benefits in treating HD. Remacemide is a glutamate antagonist that, in R6/2 mice, increases survival by 16% while improving motor functions, brain pathology, and weight loss [42]. Riluzole is a another glutamate antagonist that, in R6/2 mice, increases survival by 10% while improving motor functions, brain pathology, and weight loss [43]. In a one year clinical trial, riluzole poses a transient benefit on chorea, and a slightly more sustained effect on other motor symptoms of HD patients [44]. Memantine is an NMDA receptor antagonist that, in a two year clinical trial, seems to slow the disease progression in HD patients [45].

Apoptosis

Apoptosis is programmed cell death, mediated by activated caspases. Comparison of TUNEL staining, an indicator of apoptosis, in HD patients and control brains indicates that there is an increase in apoptosis in HD [46]. One apoptosis marker is the release of cytochrome c, which can be induced by expanded Htt [47]. Expanded Htt also activates some caspases, which may directly lead to cell death [48, 49]. In addition, Htt contains several caspase cleavage sites at the N-terminus [50] and it has been reported that expanded Htt undergoes cleavage more efficiently than normal Htt [51]. This finding may explain the abundance of N-terminal expanded Htt fragments. The size of the expanded Htt fragments inversely correlated to toxicity [52].

Several caspase inhibitors have been examined as potential HD treatments since they prevent apoptosis. The caspase inhibitor zVAD-fmk increases survival by 25% while improving motor functions, brain pathology, and weight loss in R6/2 mice [53]. Cystamine was identified as a transglutaminase inhibitor, although it seems to function in HD mice by inhibiting caspase activity [54]. In R6/2 mice, cystamine increase survival by 20% while improving motor functions, brain pathology, and weight loss [55, 56]. Minocycline inhibits activation of some caspases by preventing cytochrome c release. R6/2 mice treated with minocycline survive 14% longer while showing improvement in motor functions and brain pathology [57]. In a two year clinical trial, minocycline stabilizes both motor and neurological symptoms [58].

Toxicity

Many experiments have shown that normal Htt plays a protective role in cells. For example, turning off the expression of normal Htt in the forebrain of a conditional knock-out postnatal mouse leads to progressive neurodegeneration, behavioral abnormalities, and death [59]. In cells, expression of normal Htt protects against multiple cellular insults that would normally lead to apoptosis [60]. Normal Htt can also protect cells against the toxic effects of expanded Htt, as over-expression of normal Htt in cells with expanded Htt results in a lower incidence of cell death [61].

Mitochondrial activity

Brains of HD patients and pre-symptomatic individuals have a decrease in glucose metabolism [62]. Interestingly, mitochondrial dysfunction is found specifically in the striatum and cortex, whereas other brain areas are spared. Impaired mitochondrial activity causes ineffective metabolism of glucose which can lead to damaging release of cytochrome c into the cell [47]. Normal Htt is directly associated with mitochondrial membranes [63] and allows proper transport of mitochondria along the axon, while expanded Htt prevents it [64]. Expanded Htt also affects calcium levels, which can lead to mitochondrial dysfunction [65].

Some antioxidants mildly alleviate symptoms in HD mouse models. Alpha-lipoic acid increases survival by 7% in both R6/2 and N171-82Q mice [66]. BN82451 improves survival by 15% while improving motor functions, brain pathology, and weight loss in R6/2 mice [67]. Dichloroacetate (DCA) activates mitochondrial activity, and in both R6/2 and N171-82Q mice, it increases survival by 7% and improves motor functions, brain pathology, and weight loss [68].

Creatine is essential for normal mitochondrial function by stabilizing calcium levels and its membrane permeability, and thus prevents the release of cytochrome c. In both R6/2 and N171-82Q mice, creatine increases survival by 17% while improving motor functions, brain pathology, and weight loss [69, 70]. Creatine given to symptomatic R6/2 mice decreases the progression of symptoms, with the highest effects in younger, less symptomatic mice [71]. However, in a one year pilot clinical trial in humans, creatine had no effect on symptoms [72].

Coenzyme Q10 (CoQ10) is an essential component of the electron transport chain, and an antioxidant. In R6/2 mice, CoQ10 increases survival by 15% while improving motor functions,

brain pathology, and weight loss [42]. By combining CoQ10 with other active drugs, a higher benefit has been seen in HD mouse models. CoQ10 and minocycline in R6/2 mice increased survival by 18% [73]. CoQ10 and remacemide resulted in an increase of 32% survival of R6/2 mice [42]. However, in a 2.5 year clinical trial, CoQ10 alone or with remacemide had no effect on symptoms in humans [74].

Transcriptional deregulation

Comparison of *in situ* hybridization in R6/2 and normal mice indicates that the mRNA levels of several neurotransmitter receptors are decreased in HD [75]. Furthermore, extracts from HD patient and mouse model brains have lower mRNA levels of several Neuron Restrictive Silencer Element (NRSE)-controlled genes, including Brain Derived Neurotrophic Factor (BDNF) [76]. Normal Htt, but not expanded Htt, allows the transcription of NRSE-controlled genes by inactivating co-repressors of their transcription. One of the NRSE-responsive genes is BDNF, which is transcribed in the cortex [77]. BDNF is an important protein in neuronal activity because it acts as a neurotrophic factor, aiding in the formation or maintenance of the cortico-striatal synapse.

Microarray data from HD mouse models [78], and human brains [79] show that the transcription of many genes are decreased by the presence of expanded Htt. Expanded Htt can sequester transcription factors and prevent the transcription of their targeted genes. A notable example is CREB-Binding Protein (CBP) which is found in aggregates in HD cell and animal models, along with brains from HD patients [80, 81]. CBP contains a polyQ region, the deletion of which prevents it from being sequestered into aggregates. The expression of expanded Htt leads to decreased transcription of CBP-dependent genes [82]. Replenishing the cells with the transcription factors that have been sequestered may provide a benefit. For example, it has recently been shown that increasing the levels of CBP in an HD *Drosophila* model completely rescues neurodegeneration and transcriptional deregulation [83].

Another way expanded Htt decreases transcription is by reducing histone acetylation [84]. Acetylated histones allow the transcription machinery to access the DNA, while deacetylated histones impede this process. Several histone deacetylase inhibitors have been tested in HD animal models. SuberoylAnilide Hydroxamic Acid (SAHA) and sodium butyrate

(NaBu) rescue some of the neurodegeneration seen in an HD *Drosophila* model [85]. In R6/2 mice, SAHA alleviates some of the motor functions but has no effect on survival [86]. NaBu increases survival by 22% while improving motor functions and brain pathology in R6/2 mice [87]. Phenylbutyrate increases survival by 23% while improving brain pathology in the N171-82Q mice [88]. Another way to globally increase transcription is to reduce histone methylation, as mithramycin can do. In R6/2 mice, mithramycin increases survival by 29% while improving motor functions and brain pathology [89].

Decreased protein trafficking

Many of the proteins that are involved in protein trafficking have altered expression levels in HD [78, 79]. Normal Htt is associated with vesicle membranes, dendrites, axons, and microtubules [36] and plays a role in vesicular transport between the axon and cell body [64, 90]. BDNF is an example of a protein whose transport via the microtubules is mediated by normal Htt [91]. Expanded Htt reduces protein trafficking by sequestering many of the proteins involved in protein trafficking, as well as binding to microtubules to prevent transport.

Decreased protein degradation

Proteins tagged with ubiquitin are targeted for degradation via the proteasome, and the existence of ubiquitinated expanded Htt in aggregates suggests a dysfunction in this process [92]. The proteasome is inefficient in fully degrading ubiquitinated expanded Htt, resulting in the release of small fragments containing the polyQ region [93]. Furthermore, inhibition of the proteasome increases aggregates in a cell model [94], and prevents the clearance of expanded Htt in a conditional mouse model [95]. In addition, cells that express expanded Htt are less able to degrade Green Fluorescent Protein (GFP) tagged for proteasomal degradation [96], indicating that proteasomal dysfunction affects more than just the expanded Htt protein.

In addition to the proteasome, Htt has been shown to be degraded by lysosome-mediated autophagy. The inhibition of the proteasome seen in an HD cell model can be rescued by activation of autophagy [97]. Rapamycin sequesters mTOR which causes an activation of the autophagy degradation pathway. Rapamycin decreases neurodegeneration in an HD *Drosophila* model, and improves motor functions in an HD mouse model [98].

TOXIC FORM OF EXPANDED HTT IS STILL DEBATED

Although the loss of function of normal Htt contributes to HD symptoms by affecting several cellular processes, its loss alone is not enough to explain the extent of the dysfunctions. In a conditional mouse model, the complete loss of normal Htt in the forebrain of post-natal mice leads to only some characteristic HD symptoms [59]. However, Htt is an essential protein during development as best shown by three independently derived Htt knock-out mice, that all die in gestation [99-101]. The fact that HD patients homozygous for the disease allele survive to adulthood and do not exhibit an earlier age of onset relative to heterozygous patients [102], indicates that expanded Htt can fulfill the developmental role of normal Htt.

It must therefore be the gain of function by expanded Htt that causes most of the cellular dysfunctions. The question of which form, whether aggregated or monomeric, of expanded Htt is the toxic species is still debatable. A direct pathway to the development of therapeutic interventions aimed at specifically eliminating or reducing the formation of the toxic forms of expanded Htt protein is currently challenging, since it is unclear which form should be targeted.

Aggregates

Aggregates of expanded polyQ Htt, visible in pathological specimens in the form of inclusion bodies, are evident in diseased brains, but not control brains [6, 7]. Patients with longer polyQ regions have a higher prevalence of inclusion bodies containing aggregated Htt [103], particularly in the cortex [104]. In the nuclei of neurons, the aggregated Htt consists of small N-terminal fragments of expanded Htt, while in the cytoplasm aggregated Htt includes variably sized N-terminal expanded Htt fragments [104]. Both expanded and normal Htt are cleaved at multiple sites by caspases [50, 105], although expanded Htt is preferentially cleaved [51]. Expanded Htt nucleates into aggregates faster than normal Htt, where the rate-limiting step is believed to be a change in protein conformation [106]. In addition, the smaller the Htt fragment, the faster expanded Htt forms aggregates.

In support of the toxic aggregates theory, pure polyQ aggregates which were created *in vitro* and then introduced to cells cause toxicity when the aggregates are targeted to the nucleus [107]. However, these polyQ aggregates are very stable whereas aggregates in HD cell and animal models are dynamic. Since caspase cleavage of Htt results in the elimination of the C-

terminal Nuclear Export Signal [108], nuclear toxicity is a possible role for aggregated or monomeric expanded Htt. In addition, proteins such as chaperones, proteasome subunits, transcription regulating proteins, and ubiquitin binding proteins are sequestered by monomeric expanded Htt and found in aggregates. The loss of function of these proteins can lead to cellular dysfunction.

Some aggregation inhibitors have had some benefit in HD animal models. The compound C2-8 partially rescues neurodegeneration in an HD *Drosophila* model [109]. C4-sFv is a single-chain Fv antibody fragment that rescues larval survival and delays neurodegeneration in an HD *Drosophila* model [110, 111]. Congo Red inhibits oligomerization and, in R6/2 mice, increases survival by 16% while improving motor functions, brain pathology, and weight loss [112]. Trehalose is a disaccharide that inhibits aggregation by stabilizing proteins in a partially-folded state [113]. In R6/2 mice, trehalose increases survival by 11% while improving motor functions, brain pathology, and weight loss [114].

Monomeric

An alternative to expanded Htt aggregates having detrimental effects in cells is that aggregates benefit cells by sequestering toxic monomeric Htt and thus prevent it from interfering in cellular processes. Support for the beneficial aggregates theory comes from the fact that aggregates are mainly found in striatal and cortical cell types that are spared in HD, while those cell types that tend to die have fewer aggregates [115]. In general, aggregate density does not correlate with cell loss, as perhaps best shown in the brain of one pre-symptomatic individual which had many aggregates in the cortical region but very little cell loss [116]. Further support comes from the full rescue of motor functions, in spite of continued presence of aggregates, following arrest of expanded Htt expression in a 17 month old conditional mouse model [117]. Most recently, a compound was identified that rescues proteasome dysfunction in an HD cell model by increasing aggregate formation [118]. Direct and compelling support for the protective effect of intracellular inclusions and the toxic effects of soluble expanded Htt species is provided by time lapse cinematography of cultured neurons in an Htt model system. In this study, neurons, that show a diffuse signal for expanded Htt are more likely to die while under

observation than neurons that have aggregated expanded Htt in the form of inclusion bodies [119].

COULD REMOVAL OF DISEASE-CAUSING EXPANDED HTT BE AN OPTIMAL THERAPY FOR HD?

Drugs targeted at cellular dysfunctions or expanded Htt aggregation have not provided sufficient benefit in ameliorating HD symptoms to be considered an effective treatment. In order to evaluate the effect of removing expanded Htt after disease-onset, the conditional mouse model HD94, where exon one of expanded Htt is expressed in the forebrain, was created [120]. When the expression of expanded Htt is eliminated after onset of symptoms, the progression of motor dysfunction and neuronal loss is not only prevented but the symptoms are actually reverted. If the expression is turned off at 10 months of age, the motor dysfunction is completely rescued and aggregates are no longer visible [121]. When the expression is turned off at 17 months of age, motor dysfunction is also completely rescued, however in this case without any effect on aggregates [117].

The study of the HD94 mouse model indicates that removal of the disease-causing expanded Htt is a viable treatment for the disease which can be beneficial at multiple stages in disease progression. One way to decrease the expanded Htt protein load is to prevent its translation by the use of RNAi targeted to the striatum. RNAi treatment in N171-82Q mice results in a 50% reduction of Htt mRNA for 2 weeks, while improving motor functions [122]. In R6/1 mice, RNAi treatments cause an 80% decrease in Htt mRNA for 10 weeks, while delaying the onset of motor symptoms [123]. RNAi treatments in R6/2 mice which decreased Htt mRNA levels by about 50% for one week result in increased survival and improved motor functions, brain pathology, and body weight [124]. RNAi treatment given after onset can also prevent the progression of neuropathology [125]. These results with RNAi treatments are encouraging because they show benefit even without complete elimination of expanded Htt. However, a drawback to RNAi is that it can not be specific for expanded Htt because RNAi targets small sections of mRNA sequence, which do not differ between expanded and normal Htt. Since

normal Htt serves a protective role, it is not ideal for an HD treatment to decrease the levels of normal Htt.

An approach to HD therapy which has the potential to address the occurrence of pathology at its source would be to decrease the levels of expanded Htt while maintaining the expression of normal Htt to maximize the protective effects of normal Htt. A compound could decrease the levels of expanded Htt proteins by either reducing the efficiency of its initial production or by promoting its degradation. The elimination of expanded Htt would allow cells to recover from the cellular dysfunctions caused by the expression of expanded Htt. Such a compound would, in principle, not only prevent the onset of disease, but also stop the progression of symptoms, as seen in the HD94 mouse model [120]. A therapy which met these criteria would have no effect on the expression of normal Htt, allowing it to continue with its protective cellular functions. The fact that expanded and normal Htt have different tertiary structures [126] allows for the possibility that a chemical compound could have specificity to expanded but not normal Htt. The goal of the study presented here was to design and implement a cell-based High Throughput Screen that could identify a compound which met these criteria for an optimally effective HD therapeutic. Therefore, the assay developed is designed to target the most basic cause of HD, the expression of expanded Htt, while making no assumptions as to which cellular dysfunction causes the most harm to HD patients or which form of expanded Htt is the most toxic to cells. The results of this screen provide a proof of concept for this approach through the identification of two compounds which decrease cellular levels of expanded but not normal Htt protein. The efficacy of compounds in another cell-based assay based on the toxicity caused by expanded Htt expression further validates this approach to therapeutic intervention. These studies thus provide initial steps in a pathway towards a direct and potentially effective approach to the treatment of HD.

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CHAPTER II

DESIGN AND PRODUCTION OF A CELL-BASED HTS FOR IDENTIFYING COMPOUNDS WHICH DECREASE EXPANDED HTT PROTEIN LEVELS

ABSTRACT

Huntington's disease (HD) is a progressively degenerative neurological disorder. Individuals who inherit the IT15 gene with an expansion of the CAG repeat region inevitably succumb to motor, psychological, and cognitive symptoms. I sought to develop an assay system with the capability for identification of chemical compounds that selectively decrease the intracellular levels of disease-causing expanded polyglutamine huntingtin (Htt) protein without reducing the intracellular levels of the potentially protective normal Htt. To achieve this goal I designed a cell-based assay using the enzymatic activity of E. coli β-galactosidase as a reporter for Htt protein levels. I expressed either expanded (97Q) or normal (23Q) Htt fused to the β-galactosidase alpha-subunit (α) in an inducible fashion in PC12 cells which also expressed the β-galactosidase delta-subunit (Δ). Complementation between these expressed subunits allowed the formation of functional β-galactosidase. The level of β-galactosidase activity in these Δ-α 97Q and Δ-α 23Q cells directly correlated with the amount of α 97Q and α 23Q fusion protein levels, indicating that β-galactosidase activity could be used as a reporter in this system for Htt protein levels. Optimization of Δ-α 97Q cells for High Throughput Screening (HTS) resulted in a Z' factor of 0.73. These data demonstrate that the cell based assay system I have developed is well suited for the identification of chemical compounds which specifically decrease intracellular expanded Htt protein levels in an HTS format.

INTRODUCTION

Huntington's disease (HD) is characterized by the progressive degeneration in motor, psychological, and cognitive abilities [1, 2]. The genetic cause for this disease is an expansion of the CAG repeats in the first exon of the IT15 gene, located on chromosome 4 [3, 4]. This expansion in CAG repeats translates into an expanded poly-glutamine (polyQ) region in the huntingtin (Htt) protein. Unaffected individuals have Htt with 6-35Q (normal Htt), while HD patients have Htt with 40-100Q (expanded Htt). The variation in the polyQ expansion in patients is correlated to age of onset, where patients with a larger polyQ region have a tendency to present with symptoms earlier in life [5]. However, there is no correlation between the length of the polyQ region and the rate of disease progression.

Htt is widespread throughout the body, with the highest expression in the central nervous system [6, 7]. Expanded Htt has been implicated in decreasing mitochondrial activity [8-10], transcription [11-18], and protein degradation [19-23]. Loss of normal Htt further contributes to dysfunction in the mitochondria [9, 24], transcription [25], and protein trafficking [9, 26-28].

The ultimate end to cellular dysfunction, cell death, has also been observed in HD. Brains from both symptomatic and pre-symptomatic HD patients show a remarkable decrease in striatal and cortical neurons [29-31]. There is some evidence suggesting apoptosis in the brain as a mechanism for neuronal loss [32]. There is also evidence suggesting excitotoxicity as a specific mechanism contributing to neuronal death [33-35]. Cell-based assays expressing exon one of expanded Htt have been designed and implemented to screen for compounds that rescue toxicity [36, 37]. These screens identified several compounds which decrease toxicity, although the only class of compounds found by both screens is caspase inhibitors. An issue which must be considered in such screens is that cellular dysfunction preceding cell death may lead to many of the symptoms observed in patients. Thus, even if compounds identified by screens based on blocking cytotoxicity suppress cell death in HD patients, they may not be effective treatments for HD because they do not prevent cellular dysfunction.

The presence of inclusions containing aggregated expanded Htt in the brains of HD patients [29, 30] has led to the hypothesis that these inclusions are responsible for the cellular

dysfunction and death [38]. Many cell-based assays have been designed and implemented to screen for aggregation inhibitors, whose endpoints are directly or indirectly tied to the formation of intracellular inclusions. Several potent aggregation inhibitors have limited effects on survival of HD animal models [39-41], indicating that inhibition of aggregation may not be the most effective way to treat HD. The drawback to these aggregation screens is that they assume inclusions are responsible for HD symptoms. Since there is evidence that inclusions may be beneficial to cell survival [42], this assumption may not be correct.

Assays designed to identify compounds that target particular cellular functions, cell death, and aggregation can only be used to screen for compounds that act in one specific disease pathway. Since expanded Htt has been implicated in many cellular dysfunctions, the most straight-forward way to treat HD would be to eliminate the disease-causing expanded Htt from cells. In order to evaluate the effect of removing expanded Htt after disease-onset, the conditional mouse model HD94, where exon one of expanded Htt is expressed in the forebrain, was created [43]. When the expression of expanded Htt is eliminated after onset of symptoms, the progression of motor dysfunction and neuronal loss is not only prevented but the symptoms are actually reverted. If the expression is turned off at 10 months of age, the motor dysfunction is completely rescued and aggregates are no longer visible [44]. However, if the expression is turned off at 17 months of age, motor dysfunction is completely rescued without any effect on aggregates [45]. The study of this mouse model indicates that blockage of the production of the disease-causing expanded Htt is a viable treatment approach for the disease which can be beneficial at multiple stages in disease progression.

The first attempt at treating HD by eliminating expanded Htt was the use of RNAi treatments in various HD mouse models, which improves survival and motor functions while decreasing neuropathology [46-48]. These results with RNAi treatments are encouraging because they show benefit even without complete elimination of expanded Htt. However, a drawback to RNAi is that it can not be specific for expanded Htt because RNAi targets small sections of mRNA sequence, which do not differ between expanded and normal Htt. Since normal Htt serves a protective role, it is not ideal for an HD treatment to decrease the levels of normal Htt.

The most effective way, in principle, to treat HD is to decrease the levels of expanded Htt while maintaining the expression of normal Htt. The fact that expanded and normal Htt have different tertiary structures [49] allows for the possibility that a compound could have specificity for expanded but not normal Htt. Here, a cell-based assay was designed to identify compounds which decrease the levels of expanded but not normal Htt proteins, making no assumptions as to which cellular dysfunction causes the most harm to HD patients or which form of expanded Htt is the most toxic to cells. This assay uses β-galactosidase activity as a reporter for Htt protein levels. PC12 cells expressing the β-galactosidase delta-subunit (Δ), and either expanded (97Q) or normal (23Q) Htt fused to the β-galactosidase alpha-subunit (α) in an inducible fashion exhibit β-galactosidase activity. The level of β-galactosidase activity in these Δ-α 97Q and Δ-α 23Q cells directly correlated with the amount of α 97Q and α 23Q fusion protein levels, indicating that β-galactosidase activity could be used as a reporter for Htt protein levels. Furthermore, these cells were optimized for High Throughput Screening.

RESULTS

Assay design

A cell-based assay has been designed to identify small molecules that decrease the amount of expanded huntingtin (Htt) but do not affect the levels of normal Htt. To accomplish this task, this system exploits the sensitive and quantitative features of a Beta-galactosidase (β-galactosidase) enzymatic activity assay reported elsewhere [50, 51]. The β-galactosidase enzyme consists of two functional domains, termed the delta (Δ) and alpha (α) subunits. The β-galactosidase Δ- and α-subunits are physically separable and enzymatically inactive when expressed alone (**Fig 1A**). Restoration of functional β-galactosidase activity is mediated by a process known as alpha-complementation, successfully used in bacteriology, which occurs via intracellular association of the Δ-subunit with the highly unstable α-subunit. Rapidly degraded by cells, the small α-subunit can be stabilized when fused to Htt fragments (α Htt), which still allows for β-galactosidase activity. Thus, β-galactosidase activity is the reporter for the presence of the Htt protein in this system.

The measurement of β-galactosidase activity is highly reproducible and quantitative, making it possible for identification of compounds that decrease the protein levels of expanded Htt to varying degrees. The ideal compound for treating Huntington's disease would be one which targets expanded Htt but not normal Htt. Since in this system β-galactosidase activity depends on the expression of both the β-galactosidase Δ-subunit and α Htt fusion proteins, this ideal compound would decrease the protein levels of expanded Htt fused to the β-galactosidase α-subunit and thus abolish β-galactosidase activity (**Fig 1A**). The specificity of this compound would be identified by its ability to decrease β-galactosidase activity in cells expressing expanded Htt but not in cells expressing normal Htt.

For this assay, the β-galactosidase α-subunit was cloned upstream of the first 600 aa of Htt containing either 97Q for expanded Htt (α 97Q) or 23Q for normal Htt (α 23Q) (**Fig 1B**). This size fragment of Htt was chosen because it contains all the known caspase cleavage sites [52]. The advantage is that this fragment can undergo most if not all of the conformations that full length Htt can, thus maximizing the types of compounds that can be identified in this screen

which can differentiate between expanded and normal Htt. In order to control the timing and level of expression of both the β-galactosidase Δ-subunit and α Htt fusion proteins, constructs were cloned under the control of an ecdysone inducible system, where Ponasterone A (PonA) is used as the inducer.

Generation of stable PC12 cells

Before committing to generating stable cell lines, transient transfections were done on CHO (Chinese hamster ovary) and PC12 (rat pheochromocytoma) cells. In both cell types, β-galactosidase activity was only seen with co-transfection of the β-galactosidase Δ-subunit and either α 97Q or α 23Q constructs (data not shown). This verified that the constructs were able to undergo alpha-complementation. For stable transfections, PC12 cells were chosen because they are a pre-neuronal cell line that can be differentiated into neuronal-like cells [53, 54].

In order to make stable cell lines that express both the β-galactosidase Δ-subunit and the α Htt fusion constructs, two rounds of stable transfections were performed. In the first step, the β-galactosidase Δ-subunit was stably transfected into PC12 cells containing the expression vector for the Ecdysone Receptor, EcR PC12 cells. Small colonies of these EcR-Δ cells were individually picked and expanded. After 24 hr of induction with PonA, 3 out of 31 clones were found to alpha-complement with transiently transfected α 23Q (**Fig 2**). Clone 600-2 was chosen to continue with the next round of stable transfections because it most closely resembled the morphology of the parental cell line.

The chosen EcR-Δ cell line was then stably transfected with either the α 97Q or α 23Q to make Δ-α 97Q or Δ-α 23Q cells, respectively. Individual colonies were picked and expanded. After 24 hr of induction with PonA, 3 out of 52 Δ-α 97Q clones and 5 out of 45 Δ-α 23Q clones had β-galactosidase activity (**Fig 3**).

Selection of Δ-α 97Q and Δ-α 23Q cell lines for screen

The two rounds of stable transfections generated three Δ-α 97Q and five Δ-α 23Q cell lines that showed varying degrees of β-galactosidase activity after 24 hr of induction. Since the Δ-α 23Q cell lines were to be used as a counter-screen for the Δ-α 97Q cell line, it was important

to choose the Δ-α 97Q and Δ-α 23Q cell lines that most closely resembled each other. Therefore, multiple characteristics were examined in order to make this choice: cell size, cell morphology compared to the parental line, doubling time, percent of cells having β-galactosidase activity after induction, and amount of β-galactosidase substrate hydrolyzed (**Table 1**). Cell lines Δ-α 97Q #46 and Δ-α 23Q #44 were chosen due to their exact match in cell size, doubling time, and substrate hydrolyzed. In addition, their cell morphology and percent of cells with β-galactosidase staining were very similar. From here on, all experiments described were performed on Δ-α 97Q #46 and Δ-α 23Q #44 cells, and are referred to as simply Δ-α 97Q and Δ-α 23Q cells, respectively.

Induction with PonA correlates with β-galactosidase activity along with β-galactosidase Δ and α Htt protein levels

The β-galactosidase activity assay yields a colorimetric output, which is detected using photometry and then converted to nmoles of substrate hydrolyzed. The effects of different levels of induction of the β-galactosidase Δ-subunit and α 97Q or α 23Q proteins on β-galactosidase activity were assessed by titration of the inducer PonA (**Fig 4A**). The amount of PonA directly correlated with β-galactosidase activity. Furthermore, Western blots showed that the protein levels of β-galactosidase Δ-subunit and α 97Q or α 23Q increased with the increase in PonA (**Fig 4B and C**). These data show a direct correlation between the amount of PonA added and the levels of β-galactosidase activity detected in cell lysates, and demonstrate the sensitivity of this assay to changes in β-galactosidase Δ-subunit, α 97Q, or α 23Q protein levels.

Δ-α 97Q and Δ-α 23Q cells can degrade β-galactosidase Δ and α 97Q or α 23Q proteins

The purpose of this screen is to identify compounds that can decrease the protein levels of expanded Htt but not normal Htt. One likely mechanism to accomplish this would be to promote the degradation of the proteins. Therefore, it was important to show that Δ-α 97Q and Δ-α 23Q cells were capable of degrading the induced α 97Q or α 23Q proteins on their own. This was assessed by inducing cells with PonA for 24 hr. At this point, the medium was removed from all wells and replaced with either medium without PonA (washed) or medium

with PonA (un-washed). By 12 hr after removal of the inducer, the β-galactosidase activity was the same as un-induced (0 hr) cells **(Fig 5A and B)**. At this time, the induced α 97Q and α 23Q protein levels dramatically decreased, as well as the induced β-galactosidase Δ-subunit protein levels, although less so. By 24 hr after the removal of the inducer, Western blots show no detectable α 97Q and α 23Q **(Fig 5C and D)** or β-galactosidase Δ proteins **(Fig 5E and F)**. This shows that the lack of β-galactosidase activity in the screen at 24 hr is an appropriate indication of degradation of the α 97Q or α 23Q fusion proteins and/or the β-galactosidase Δ-subunit.

Optimization of β-galactosidase assay for High Throughput Screen

It is important to maximize speed and reproducibility in a High Throughput Screen (HTS). Therefore, several aspects of the β-galactosidase assay were examined in order to find the protocol which minimized the number of steps while maximizing the enzymatic read-out (data not shown). The number of cells seeded per 96-well was maximized to ensure a high β-galactosidase activity without over-seeding which could result in unhealthy cells. It was determined that only a single PBS wash was needed to fully remove the media from the wells to ensure reproducible results. Multiple recipes of lysis buffer were tested to find one which minimized the background absorbance reading at 405 nm, maximized the detection of β-galactosidase activity, and minimized the amount of time needed for maximal β-galactosidase activity. It was determined that 30-45 min in 37°C resulted in an absorbance reading at 405 nm near but not above 1.0, which is the limit of reliability. Finally, addition of STOP buffer after this incubation allowed the β-galactosidase signal to remain stable, giving flexibility in the timing of plate reading.

Δ-α 97Q cell population has high Z' factor

These experiments showed that Δ-α 97Q and Δ-α 23Q cells are capable of reporting what they were designed to do, namely use β-galactosidase activity as a reporter for the protein levels of α Htt. However, in order to be useful for an HTS, the β-galactosidase activity of the negative and positive controls had to be significantly different for statistical analysis of the effect of compounds. One way to measure this is with Z' factor, which takes into account the difference

of the mean between two populations and the variance within each population (**Fig 6**). A Z' factor higher than 0.2 is considered acceptable for HTS, while higher than 0.5 is desirable. Using the optimized protocol for HTS, the Z' factor for Δ-α 97Q cell population was calculated to be 0.73 under conditions for use in Aleksey Kazantsev's HTS laboratory at Massachusetts General Hospital. These data indicate that Δ-α 97Q cells are highly suitable for HTS.

DISCUSSION

Although Huntington's disease is known to be caused by the expression of the Htt protein with expanded glutamines, it is still widely debated how this protein causes the vast spectrum of symptoms in patients. Which of the cleaved Htt-fragments is most detrimental? Which role of expanded Htt within a cell is the disease-causing role and which roles are non-consequential? Does the disease mechanism best correlate with cellular dysfunction or cell death? While these are all important and scientifically relevant questions to answer, designing a cell-based drug screen founded on a potential disease-causing role for Htt is risky. For this reason, a drug screen was designed based on the fact that the expression of expanded Htt is detrimental to cells, making no assumptions as to which form or role of expanded Htt is responsible for the disease mechanism.

The complexity of symptoms of Huntington's disease is a combination of gain-of-function by expanded Htt and loss-of-function of normal Htt. For this reason, a counter-screen was designed with normal Htt to rule out compounds that also decrease the protein levels of normal Htt. This distinction is important because normal Htt has been shown to be protective in cells [55-57]. The fact that expanded and normal Htt have different tertiary structures [49] allows for the possibility of a compound to selectively recognize expanded but not normal Htt.

The cellular assay described here was designed using β-galactosidase activity as a reporter for the presence of expanded (97Q) or normal (23Q) Htt. Two cell lines were generated, Δ-α 97Q and Δ-α 23Q, under an ecdysone inducible system. Induction with PonA directly correlated with β-galactosidase activity, and the protein levels of β-galactosidase Δ-subunit and α 97Q or α 23Q. In addition, the cells were able to degrade these induced proteins within 24 hr after removal of inducer. Together, these sets of experiments show that the amount of β-galactosidase activity (i.e., of β-galactosidase substrate cleaved) correlates with the amount of α 97Q or α 23Q present in cells and serves as an indicator of Htt clearance. Furthermore, these results show that these cells are capable of clearing expanded Htt, recapitulating the clearance observed in the inducible mouse model [43] of HD. This confirms that the cell lines possess an

intrinsic mechanism for degrading expanded Htt, and validates this cell line for use in an HTS to identify small molecules.

While this screen was designed to use β-galactosidase activity as a reporter for the decrease in protein levels of expanded Htt, the loss of β-galactosidase activity in this assay can be caused by a number of different mechanisms. The compounds I particularly sought would decrease the amount of expanded Htt protein, but not normal Htt. The decrease in protein may be accomplished either in the production (transcription, translation) or degradation of protein. The degradation of proteins can be increased by proteasome-targeting or affecting chaperones to increase misfolding. The identification of a compound which differentially acted preferentially on expanded Htt would be of particular significance because it would represent proof of principle that this approach to therapeutic intervention in HD was technically feasible.

Regardless of the mechanism by which a compound specifically decreases levels of expanded but not normal Htt, such a compound should also score positively in assays which measure downstream consequences of expanded Htt function, including the inhibition of aggregation and the rescue from toxicity in other cellular assays. Compounds of this type may be helpful in sorting out the relationships among theories regarding which products of expanded Htt are most significant in causing pathology.

If aggregates are toxic to cells, then reduction in aggregates would lead to a reduction in cell death. Evidence to support the toxic aggregate theory comes from experiments where pure polyQ aggregates taken up by cells causes toxicity when the aggregates are targeted to the nucleus [58]. Since caspase cleavage of Htt results in the elimination of the C-terminal nuclear export signal [59], nuclear toxicity is a possible role for aggregated expanded Htt. In addition, sequestered proteins such as chaperones, proteasome subunits, transcription regulating proteins, and ubiquitin binding proteins are found in aggregates. The loss of function of these proteins can lead to cellular dysfunction, and ultimately cell death.

If the alternative theory that monomeric expanded Htt is toxic because it interferes in cellular processes is true, then compounds that decrease levels of expanded but not normal Htt protein would rescue toxicity because they eliminate the disease-causing protein. Support for the toxic monomeric theory lies in the observation that neurons which show a diffuse signal for

expanded Htt are more likely to die than neurons that have aggregated expanded Htt [60]. In this theory, aggregates are actually protective because they sequester monomeric expanded Htt and prevent it from doing further harm in a cell. Further support comes from the observation that aggregates are mainly found in striatal and cortical cell types that are spared in HD, while those cell types that tend to die have fewer aggregates [61]. In general, aggregate density does not correlate with cell loss [62]. This is perhaps best shown in the brain of one pre-symptomatic individual which had many aggregates in the cortical region but very little cell loss. Further support comes from the full rescue of motor functions, in spite of continued presence of aggregates, following arrest of expanded Htt expression in a conditional mouse model [45]. Most recently, compounds have been identified that rescue proteasome dysfunction in an HD cell model by increasing aggregate formation [63].

Compounds identified in the Δ-α 97Q screen described here could fall into another category, where the decrease in β-galactosidase activity is caused by affecting the alpha-complementation specifically in Δ-α 97Q but not Δ-α 23Q cells. Possible mechanisms would be physical interference between the β-galactosidase subunits, alteration of expanded Htt conformation, or re-localization of expanded Htt to a different cellular compartment. All of these could prevent alpha-complementation between the β-galactosidase Δ-subunit and α 97Q proteins. While these mechanisms themselves may not benefit the treatment of the disease, these compounds could still be interesting because of their selectivity to expanded Htt but not normal Htt. Study of these compounds could lead to knowledge of how they confer selectivity for expanded Htt, which could be applied to other compounds that do promote degradation.

The final category of compounds that could be identified in this screen is one where compounds are affecting common components of the two cell lines. These would be compounds which decrease β-galactosidase activity in both Δ-α 97Q and Δ-α 23Q cells by decreasing the number of cells, decreasing any Htt protein levels, decreasing β-galactosidase Δ-subunit levels, or interfering with the ecdysone inducible system (preventing either PonA from binding to the ecdysone receptor, or the ecdysone receptor from binding to E/GRE). While compounds that decrease the protein levels of both expanded and normal Htt are not the focus of this screen, these compounds could still be beneficial for drug development since medicinal chemistry may

create a related compound that maintains the ability to decrease Htt protein levels, but makes it specific to expanded Htt. However, the Δ-α 23Q counter-screen would be unable to differentiate between compounds that decrease the protein levels of all Htt proteins and those that affect common components between the two cell lines. Therefore, additional experiments would be necessary to determine this information, discussed in Chapter IV.

MATERIALS AND METHODS

Cloning

The β-galactosidase Δ-subunit, lacking the first 500 bp of β-galactosidase, was cloned into a pIND vector (Invitrogen), which contains five E/GREs and a promoter for induction with Ponasterone A. The β-galactosidase α-subunit, the first 500 bp of β-galactosidase, was cloned upstream of the first 600 aa of Htt containing either 97Q or 23Q, with a FLAG-tag in between. These constructs, α 97Q and α 23Q, were then cloned into a pIND/Hygro vector (Invitrogen), containing five E/GREs and a promoter for induction with Ponasterone A.

Generation of stable PC12 cell lines

PC12 cells stably transfected with pVgRXR (Ecdysone Receptor expression vector), was used as the parental cell line and is referred to as EcR PC12 (gift from Aleksey Kazantsev). Lipofectamine 2000 (Invitrogen) was used to stably transfect β-galactosidase Δ-subunit in pIND and α 97Q or α 23Q in pIND/Hygro into the EcR PC12 cells in two steps: 1. β-galactosidase Δ-subunit; 2. α 97Q or α 23Q. These steps generated Δ-α 97Q and Δ-α 23Q PC12 cell lines, respectively.

Cell culture

Δ-α 97Q and Δ-α 23Q PC12 cells were grown in DME medium with 15% fetal bovine serum, 2 mM penicillin-streptomycin, 2 mM L-glutamine at 37°C with 5% CO_2. The pVgRXR, pIND β-galactosidase Δ-subunit, and pIND/Hygro α 97Q or α 23Q constructs were maintained with 0.2 mg/ml Zeocin, 0.25 mg/ml Geneticin, and 0.1 mg/ml Hygromycin, respectively.

β-galactosidase activity assay

Either Δ-a polyQ cells were seeded at 50 x 10^4 cells/ml in 96-well format, induced with 5 μM Ponasterone A (AG Scientific) in DMSO, and then grown at 37°C for 24 hr. Each well was then rinsed with PBS, and 10 μl of Modified RIPA added (150 mM NaCl, 50 mM Tris HCl pH 7.4, 1 mM EDTA, 1% NP-40, 1% w/v Na-deoxycholate, stored at 4°C). 67 μl of a master mix (5 μl 10x Cleavage Buffer, 0.135 μl 14.3 M β-mercaptoethanol, 44.865 μl dH_2O, 17 μl 4 mg/ml ONPG) from Invitrogen's β-galactosidase Assay Kit was added to each well and incubated at 37°C for 30-60 min. The addition of 125 μl of STOP Buffer (1 M Na_2CO_3)

stabilizes the colorimetric change of ONPG cleaved by β-galactosidase, which was then read at 405 nm on a plate reader.

β-galactosidase activity is reported in nmoles of ONPG hydrolyzed. The absorbance at 405 nm can be converted to nmoles of ONPG hydrolyzed by the following formula:

$$\text{nmoles of ONPG hydrolyzed} = \frac{(\text{OD @ 405 nm}) * (\text{final vol} = 1.92 \times 10^5 \text{ nl})}{(4500 \text{ nl/nmole-cm}) * (1 \text{ cm})} = (OD_{405nm}) * (42.667 \text{ nmole})$$

The data from the experimental wells was normalized by subtracting the amount of β-galactosidase activity in un-induced wells.

Western Blots

Either Δ-α 97Q or Δ-α 23Q cells were seeded in 12-well plates at 50 x 10^4 cells/ml and grown at 37°C for 24 hr. Proteins were extracted from cells with Lysis Buffer (50 mM Tris pH8, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40) and Complete Protease Inhibitors (Roche) at 2x, on ice 30 min. Protein concentration was determined using Protein Assay and BSA standards (Bio Rad). 10-15 μg of protein in SDS loading buffer with B-mercaptoethanol was heated at 80°C for 5 min, then loaded on an 8.5% acrylamide gel in a PROTEAN II system (Bio Rad). Proteins were transferred to PVDF (Millipore) using 15% MeOH in transfer buffer (25 mM Tris, 190 mM glycine), at 80 V for 2 hr in 4°C.

PVDF blots were blocked in PBST with 0.5% milk for 1 hr. Actin (1:500, Sigma), β-galactosidase (1:2000, MP Bio), or MAB2166 Htt (1:2000, Chemicon) antibodies were diluted in PBST with 0.5% milk and incubated for 2 hr at room temperature or overnight at 4°C. HRP-conjugated secondary antibodies were diluted in PBST with 0.5% milk and incubated with blots for 30-45 min. Proteins were visualized with ECL Plus (Amersham Biosciences), and blots exposed to MR film (Kodak).

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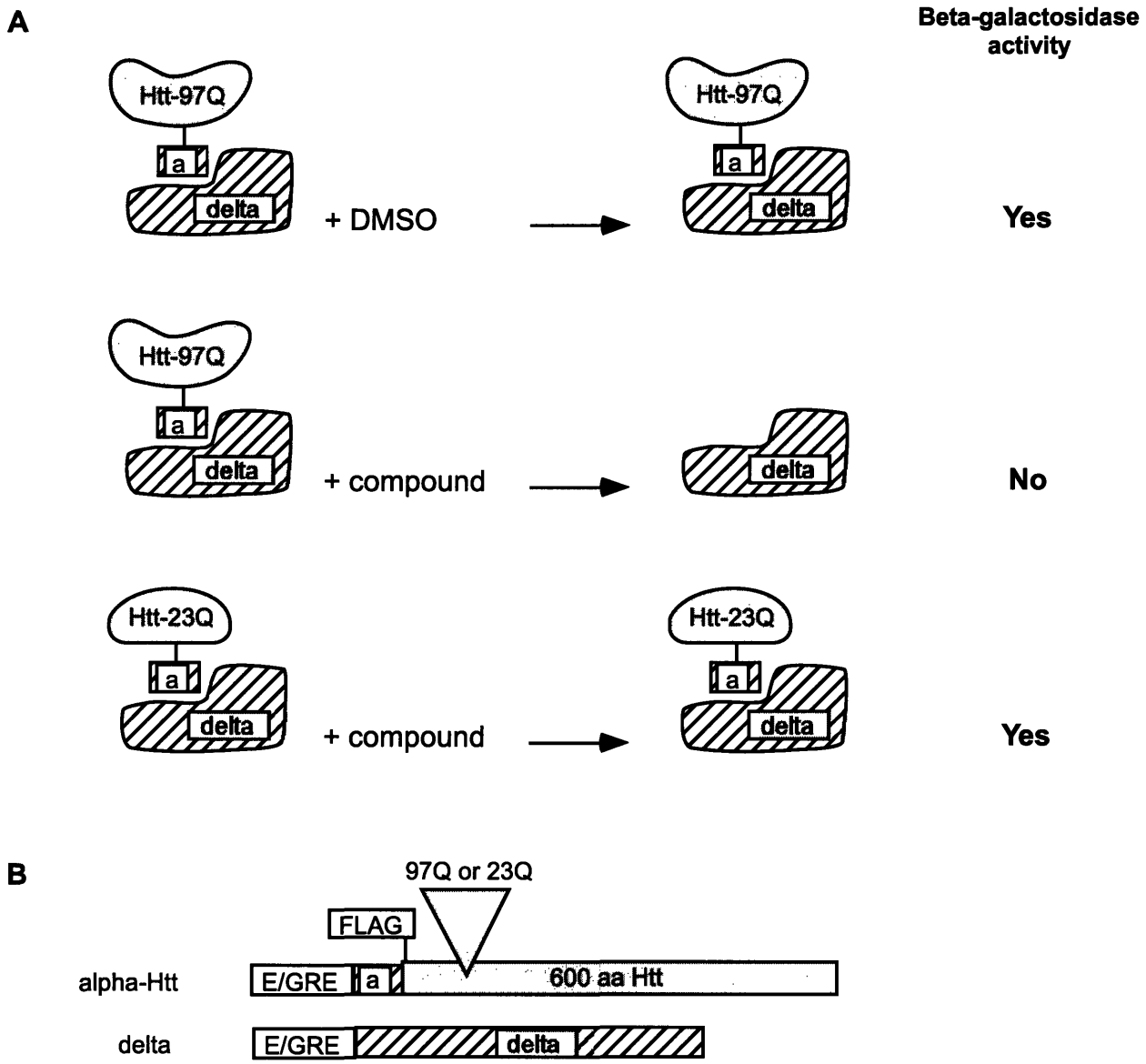

Figure 1: Beta-galactosidase activity as reporter assay for presence of Htt
A) The expression of neither the Beta-galactosidase alpha-fusion or delta-subunit is sufficient for Beta-galactosidase activity. Co-expression of the alpha-fusion and delta-subunit will re-constitute the Beta-galactosidase activity. The ideal compound is one which specifically recognizes expanded Htt-97Q and promotes its degradation but does not affect the level of normal Htt-23Q. This compound can be identified in the assay because it will degrade the expanded Htt which is fused to the alpha-subunit. The elimination of the alpha-subunit will eliminate Beta-galactosidase activity.
B) Inducible PC12 cell lines express the Beta-galactosidase delta-subunit and the Beta-galactosidase alpha-subunit fused to the first 600 aa of Htt, containing either 23Q or 97Q. Both constructs are under the expression of the ecdysone inducible system.

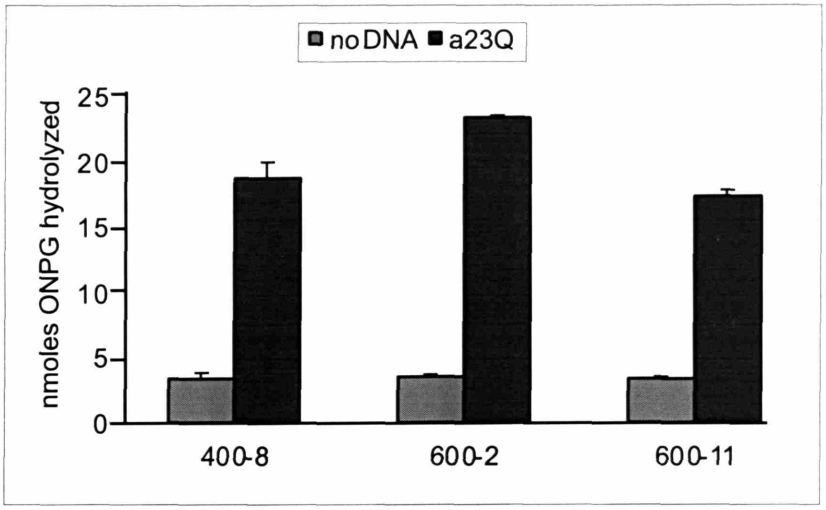

Figure 2: Stable EcR-delta PC12 cell lines identified.
EcR PC12 cell lines stably transfected with the Beta-galactosidase delta-subunit were transiently transfected with a23Q, and subsequently induced with 5 uM PonA for 24 hr. Beta-galactosidase enzymatic activity of each cell line was detected by the Invitrogen Beta-galactosidasel Assay Kit and reported as nmoles of ONPG (B-galactosidase substrate) hydrolyzed. Error bars indicate standard deviation. N=3.

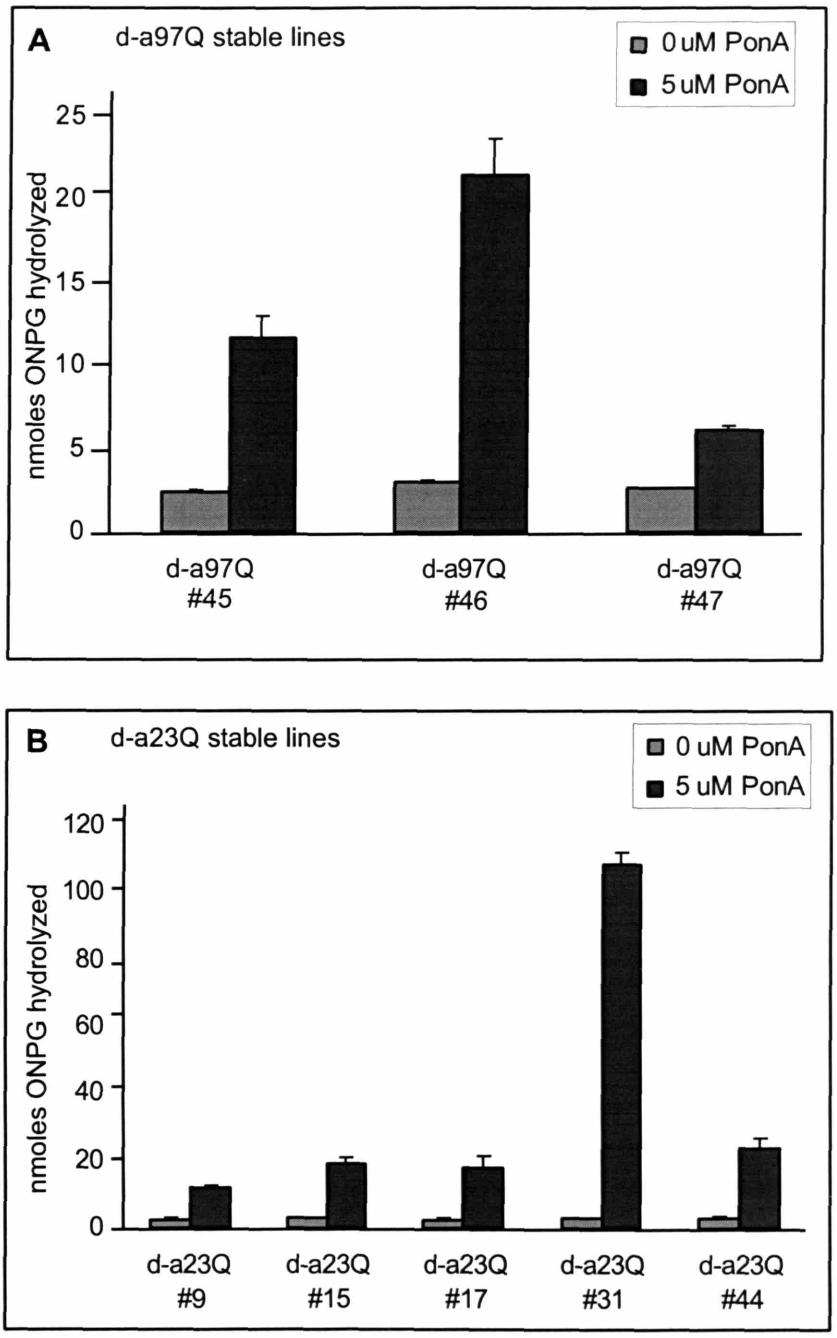

Figure 3: d-a97Q and d-a23Q PC12 cell lines are identified.
delta PC12 cell lines stably transfected with either a97Q (**A**) or a23Q (**B**) were induced with 5 uM PonA for 24 hr. Beta-galactosidase enzymatic activity of each cell line was detected by the Invitrogen Beta-galactosidase Assay Kit and reported as nmoles of ONPG hydrolyzed. Error bars indicate standard deviation. N=12.

	cell size	cell morphology (vs. parental)	doubling time	Beta-galactosidase staining	nmoles ONPG hydrolized
d-a97Q #44	small	some different	1.5 days	10%	9.2
#46	small	same	>1day	> 50%	18.5
#47	small	same	<1 day	10%	3.5
d-a23Q #9	smallish	same	1 day	50%	9.2
#15	larger	some different	1 day	> 50%	16.12
#17	larger	some more different	1 day	25%	15.6
#31	small	same	1 day	> 75%	92.9
#44	small	some different	1.5 day	< 50%	20.9

Table 1: Comparison of d-a97Q and d-a23Q cell lines.
The three d-a97Q (#45, #46, #47) and five d-a23Q (#9, #15, #17, #31, #44) cell lines were examined on various parameters in order to choose the most ideal d-a97Q cell line for HTS, and the best d-a23Q cell line to use as a counter screen for compound specificity. Cell size and the amount of seemingly differentiated cells are comparative measures. Doubling time was determined by a standard growth curve. The percent of cells with Beta-galactosidase enzymatic activity was determined by inducing the cells with 5 uM PonA for 24 hr and subsequently staining for Beta-galactosidase activity using Invitrogen's Bgal Staining Kit. The total nmoles of ONPG that a population of cells is capable of hydrolyzing was used as a measure of Beta-galactosidase enzymatic activity, with Invitrogen's Beta-galactosidase Assay Kit. This was determined by subtracting the nmoles of ONPG hydrolyzed in un-induced cells from the nmoles of ONPG hydrolyzed in cells induced with 5 uM PonA for 24 hr.

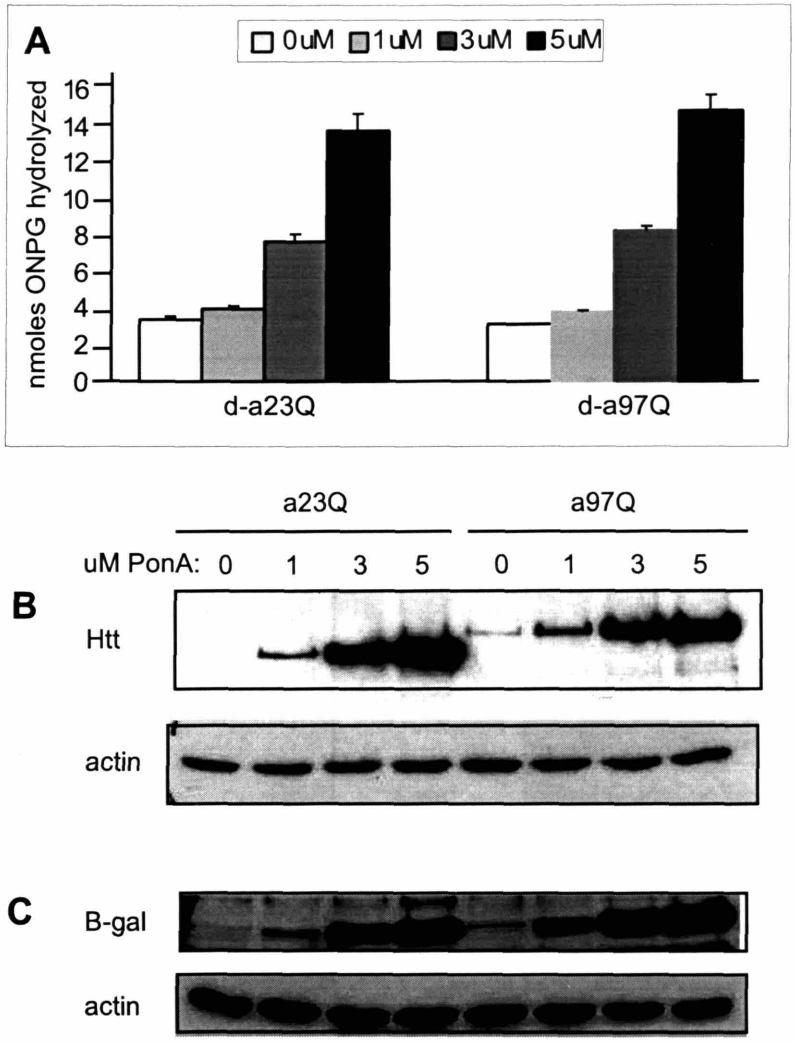

Figure 4: Induction with PonA directly correlates with Beta-galactosidase activity and induced protein levels.
d-a23Q or d-a97Q cells were either untreated (0 uM) or induced with 1, 3, or 5 uM Ponasterone A (PonA) for 24 hr. Cells were either assayed for Beta-galactosidase activity using the Invitrogen's Beta-galactosidase Assay Kit (**A**) or lysed for Western blot analysis (**B, C**). **A**) Higher concentrations of PonA result in more Beta-galactosidase activity, as determined by nmoles of substrate hydrolyzed. Error bars indicate standard deviation. N=5. **B**) Western blot analysis was used to detect the expressions of a23Q or a97Q and actin using the MAB2166 Htt and Actin antibodies, respectively. **C**) The detection of the Beta-galactosidase delta-subunit and Actin was achieved by the Beta-galactosidase and Actin antibodies, respectively.

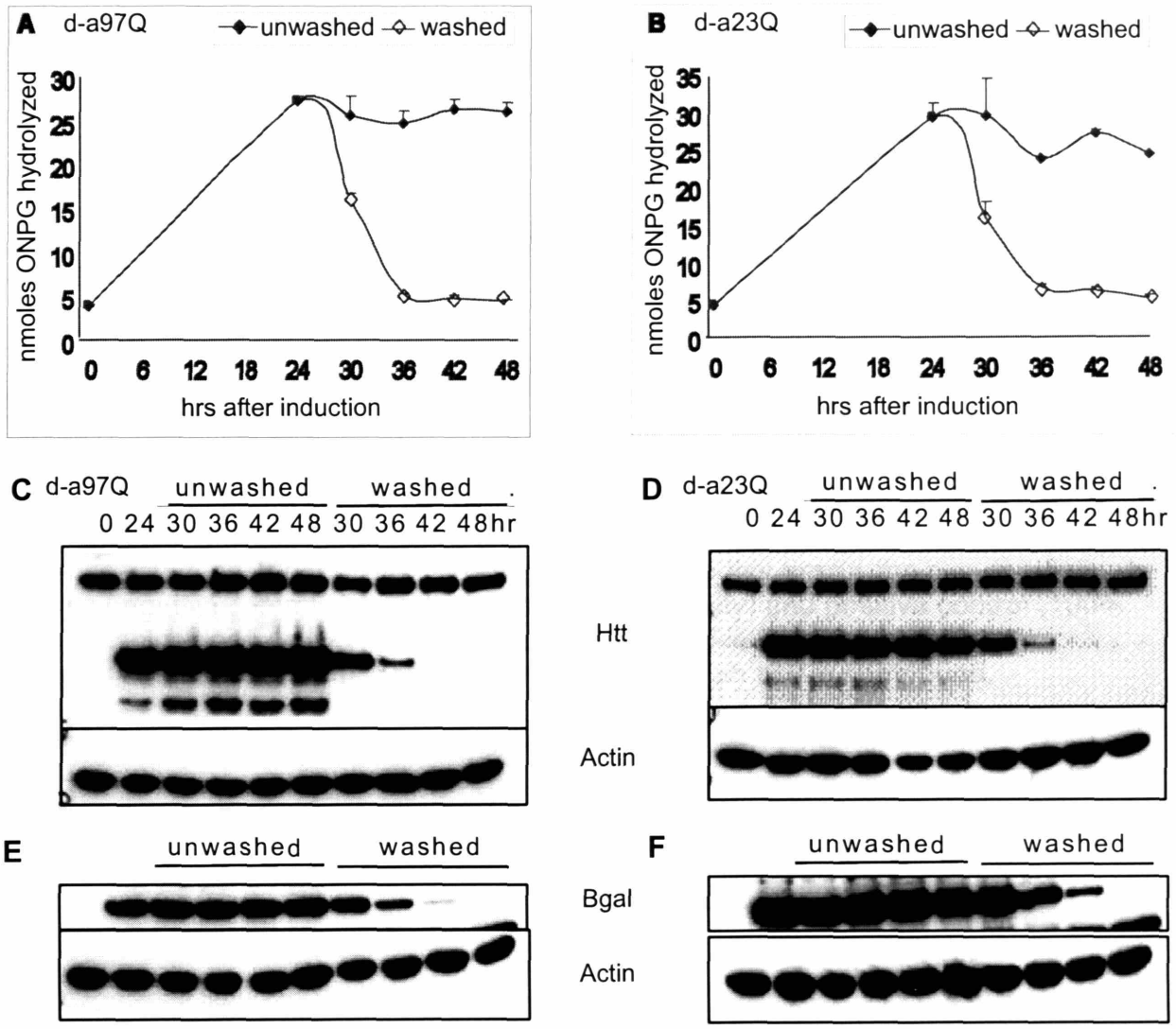

Figure 5: Decrease in Beta-galactosidase activity correlates with reduction of induced protein levels.

d-a97Q and d-a23Q cells were induced with 5 uM PonA. After 24 hr, the media of each well was replaced either with media containing PonA (unwashed) or media without inducer (washed). Cells were either assayed for Beta-galactosidase activity (**A, B**) or lysed for Western blot analysis (**C-F**) at the following time points: 0, 24, 30, 36, 42, or 48 hr. d-a97Q data shown in **A, C, E**; d-a23Q data in **B, D, F**. **A, B**) The Beta-galactosidase activity of continuously induced cells (closed) and of washed cells (open) was determined using the Invitrogen Beta-galactosidase Assay Kit. Error bars indicate standard deviation. **C, D**) Western blot analysis using MAB2166 Htt and Actin antibodies to detect the a-Htt fusion expression and Actin, respectively. Upper bands are inducible a-Htt fusion protein at full length, and lower bands are cleaved products of a-Htt fusion protein. **E, F**) Western blot analysis using the Beta-galactosidase and Actin antibodies.

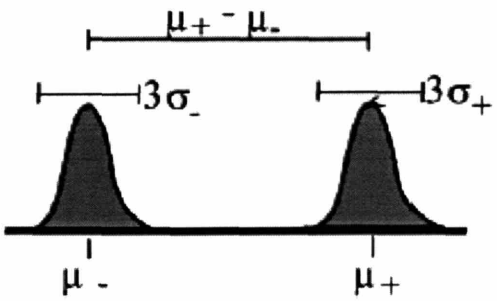

Figure 6: Z' Factor description
To accurately detect a shift in the mean of a population towards another population, the two populations would ideally have a large distance between their means, and the variance within each population would be minimal. In this assay, the two populations are the negative control (0% Beta-galactosidase activity) and positive control (100% Beta-galactosidase activity). The shift being detected is the change in Beta-galactosidase activity from 100% with the addition of an active compound.

CHAPTER III

IDENTIFICATION AND CHARACTERIZATION OF COMPOUNDS THAT DECREASE EXPANDED HTT PROTEIN LEVELS

ABSTRACT

Huntington's disease (HD) is a progressively degenerative neurological disorder. Individuals who inherit the IT15 gene with an expansion of the CAG repeat region inevitably succumb to motor, psychological, and cognitive symptoms. I sought to identify chemical compounds that specifically caused a decrease in intracellular levels of the disease-causing expanded huntingtin (Htt) protein without reducing the levels of the protective normal Htt. In this chapter, I describe the identification of compounds which meet these criteria, a proof of principle for this approach. I further show that these compounds are effective in reducing toxicity caused by expanded Htt in a cell based model system further validating this approach to HD therapy. To carry out these studies I implemented a cell-based assay, using β-galactosidase activity as a reporter for Htt protein levels, as a secondary assay to characterize a group of 34 compounds initially identified in a High Throughput Screen format because they reduced levels of expanded Htt fused to GFP. Of the 34 compounds characterized in the β-galactosidase Δ-α 97Q assay, dose response curves for 11 compounds verified their activity at 10 μM or less. Counter-screening of these 11 compounds with Δ-α 23Q cells revealed that seven compounds decrease β-galactosidase activity only in Δ-α 97Q, and four compounds decrease β-galactosidase activity in both cell lines. Immunofluorescence further demonstrates that two of the compounds decrease levels of expanded but not normal Htt proteins in the cells. Finally, tests of toxicity on Htt^{Q103} PC-12 cells, which show specific toxicity following expression of an expanded Htt protein, revealed a significant correlation with the results from the β-galactosidase assay and the identification of at least one compound which continued to meet the criteria for therapeutic intervention in HD. These results support the feasibility of the development of an HD therapeutic strategy based on small molecules which cause a specific reduction of intracellular Htt expanded repeat protein levels and suggest a program of development for such molecules.

INTRODUCTION

Huntington's disease (HD) is characterized by the progressive degeneration in motor, psychological, and cognitive abilities [1, 2]. The genetic cause for this disease is an expansion of the CAG repeats in the first exon of the IT15 gene, located on chromosome 4 [3, 4]. This expansion in CAG repeats translates into an expanded poly-glutamine (polyQ) region in the huntingtin (Htt) protein. Unaffected individuals have Htt with 6-35Q (normal Htt), while HD patients have Htt with 40-100Q (expanded Htt). The variation in the polyQ expansion in patients is correlated to age of onset, where patients with a larger polyQ region have a tendency to present with symptoms earlier in life [5]. However, there is no correlation between the length of the polyQ region and the rate of disease progression.

Htt expression is widespread throughout the body, with the highest levels of expression in the central nervous system [6, 7]. Expanded Htt has been implicated in decreasing mitochondrial activity [8-10], transcription [11-18], and protein degradation [19-23]. Loss of normal Htt can further contribute to dysfunction in mitochondrial activity [9, 24], transcription [25], and protein trafficking [9, 26-28]. Cell death has also been observed in HD. Brains from both symptomatic and pre-symptomatic HD patients show a remarkable decrease in striatal and cortical neurons [29-31]. Evidence of apoptosis in the brains is one mechanism of cell loss [32], while excitotoxicity also contribute [33-35]. Nuclear inclusions containing Htt protein are observed in surviving neurons in the brains of HD patients at autopsy. However, the pathological role of such inclusions or their potentially beneficial role in cell survival remains a question of significance [36].

Understanding of the dysfunctional cellular processes has lead to potential drug therapies, in particular those targeting the mitochondria [37-44], transcription [18, 45-49], protein degradation [50], and toxicity [51-60]. However, none of these drug treatments are effective enough to be considered a viable HD treatment. One possible reason is that each of these drugs target only one aspect of HD, leaving the possibility that symptoms arising from other dysfunctions would persist.

Alternative drugs have been targeted at aggregation, based on the proposal that the toxicity caused by aggregated Htt is the cause of the cellular dysfunction. Support for this theory comes from the presence of aggregated expanded Htt in the brains of HD patients [29, 30]. Pure polyQ aggregates taken up by cells causes toxicity when the aggregates are targeted to the nucleus [61]. Since caspase cleavage of Htt results in the elimination of the C-terminal nuclear export signal [62], nuclear toxicity is a possible role for aggregated expanded Htt. In addition, sequestered proteins such as chaperones, proteasome subunits, transcription regulating proteins, and ubiquitin binding proteins are found in aggregates. The loss of function of these proteins can lead to cellular dysfunction, and ultimately cell death. However, several potent aggregation inhibitors have limited effects on survival of HD animal models [63-65], indicating that inhibition of aggregation may be of limited effectiveness in treating HD.

Since expanded Htt, aggregated or not, has been implicated in many cellular dysfunctions, a direct way to treat HD would be to eliminate the disease-causing expanded Htt from cells. In order to evaluate the effect of removing expanded Htt after disease-onset, the conditional mouse model HD94, where exon one of expanded Htt with 94Q is expressed in the forebrain, was created [66]. When the expression of expanded Htt is eliminated after onset of symptoms, the progression of motor dysfunction and neuronal loss is not only prevented but the symptoms are actually reverted. If the expression is turned off at 10 months of age, the motor dysfunction is completely rescued and aggregates are no longer visible [67]. However, if the expression is turned off at 17 months of age, motor dysfunction is completely rescued without any effect on aggregates [68]. The study of this mouse model indicates that removal of the disease-causing expanded Htt is a viable treatment for the disease which can be beneficial at multiple stages in disease progression.

The first attempt treating HD by eliminating expanded Htt was the use of RNAi treatments in various HD mouse models, which improves survival and motor functions while decreasing neuropathology [69-71]. These results with RNAi treatments are encouraging because they show benefit even without complete elimination of expanded Htt. However, a drawback to RNAi is that it can not be specific for expanded Htt because RNAi targets small sections of mRNA sequence, which do not differ between expanded and normal Htt. Since

normal Htt may serve a protective role, it is not ideal for an HD treatment to decrease the levels of normal Htt.

An approach to the treatment of HD which, in principle, would be extremely attractive would be to identify treatments that decrease the levels of expanded Htt while maintaining the expression of normal Htt. The fact that expanded and normal Htt have different tertiary structures [72] allows for the possibility that a chemical compound could have specificity to expanded but not normal Htt. Here, a cell-based assay was implemented to identify compounds that decrease the levels of expanded but not normal Htt proteins, making no assumptions as to which cellular dysfunction causes the most harm to HD patients or which form of expanded Htt is the most toxic to cells. This assay uses β-galactosidase activity as a reporter for Htt protein levels. PC12 cells expressing the β-galactosidase delta-subunit (Δ), and either expanded (97Q) or normal (23Q) Htt fused to the β-galactosidase alpha-subunit (α) in an inducible fashion exhibit β-galactosidase activity. These Δ-α 97Q cells were used to screen compounds in a High Throughput Screen format. Counter-screening with Δ-α 23Q cells and secondary assays identified two compounds which decrease levels of expanded but not normal Htt proteins. These data indicate that the Δ-α 97Q cells and Δ-α 23Q counter-screen are effective in identifying compounds that selectively decrease expanded Htt protein levels serving as a proof of principle for this approach to HD therapy.

RESULTS

Validation of assay and identification of 11 effective compounds

Δ-α 97Q PC12 cells were constructed as a cell-based assay to identify compounds that decrease the level expanded Htt protein. The cell line is stably transfected with two constructs: one encodes the Beta-galactosidase (β-galactosidase) delta-subunit (Δ), and the other encodes a fusion between the first 600 aa of expanded Htt (with 97Q) and the β-galactosidase alpha-subunit (α). Thus, Htt protein levels are monitored by β-galactosidase activity due to alpha-complementation of β-galactosidase Δ-subunit and α-subunit fused to Htt (α 97Q). Both the β-galactosidase Δ-subunit and α 97Q proteins are under the control of the inducible ecdysone system and can be expressed upon the addition of the inducer Ponasterone A (PonA). A similar cell line containing a normal length of the polyQ region, Δ-α 23Q, was also created to be used as a counter-screen to identify compounds that specifically decrease the protein levels of expanded but not normal Htt.

In order to validate Δ-α 97Q cells as capable of identifying compounds which decrease β-galactosidase activity by decreasing α 97Q protein levels, a collection of compounds previously shown to decrease an N-terminal Htt fragment (Htt 1-17 aa) with 103Q fused to EGFP was tested. Aleksey Kazantsev previously identified 114 compounds that decreased the overall levels of EGFP (**Fig 1A**) [73]. Since these PC12 cells expressed only a small Htt fragment with 103Q, any specific compound that affected the levels of EGFP was presumably targeting the polyQ. However, some compounds might act non-specifically, for example by disrupting transcription, ecdysone induction, or cell viability. Therefore, there was a distinct likelihood that some compounds in this collection would be capable of decreasing β-galactosidase activity in Δ-α 97Q cells.

Kazantsev's lab tested these 114 compounds on the Δ-α 97Q cells in a High Throughput Screen (HTS) format. Of the 114 compounds tested, 34 compounds showed less than 75% β-galactosidase activity as compared to induced cells without compound and were considered hits. These 34 compounds were re-tested for validation by performing dose response tests on Δ-α 97Q cells in a low-throughput manner. At 10 μM, 17 compounds were found to have more

than 101% β-galactosidase activity, five compounds with 76-100% β-galactosidase activity, and one could not provide reproducible results (**Fig 1B**). These 23 compounds were considered false positive compounds and were not further analyzed. The remaining 11 compounds showed linear dose response, and at 10 μM had less than 75% β-galactosidase activity. Specifically, one compound had 0-25% β-galactosidase activity, five had 26-50% β-galactosidase activity, and five had 51-75% β-galactosidase activity.

Four classes of compounds identified by Δ-α 23Q counter-screen

The 11 compounds that caused 0-75% β-galactosidase activity in Δ-α 97Q cells were then tested in Δ-α 23Q cells by dose response curves to determine their specificity to expanded Htt. Upon examination of the data, the compounds were categorized into four classes (**Table 1**). Class I is composed of compounds that decrease β-galactosidase activity in Δ-α 97Q cells, but increase it in Δ-α 23Q cells as compared to induced cells without compound. The Class II compound has complex effects on both cell lines. Class III compounds decrease β-galactosidase activity in Δ-α 97Q cells but have no effect on Δ-α 23Q cells. Finally, Class IV compounds decrease β-galactosidase activity in both cell lines. Classes I, II, and III are compounds that have specificity to expanded Htt, and are described below. Class IV compounds are non-specific, and will be described in a later section.

Characterization of seven specific acting compounds:

Seven specific acting compounds are grouped into Classes I, II, and III

Compounds A7, A8, A9, and A24 were placed in Class I because between 1-10 μM, none caused the β-galactosidase activity of Δ-α 97Q to rise above 125%, while the β-galactosidase activity of Δ-α 23Q does increase (**Fig 2**). These are the most promising compounds because if β-galactosidase activity is a true measure for either α 97Q or α 23Q proteins, then these compounds have the ability to decrease the level of expanded Htt and increase the level of normal Htt.

Compound A18 stands alone in Class II with its ability to dramatically increase β-galactosidase activity in both Δ-α 97Q and Δ-α 23Q cells at low concentrations (**Fig 3**).

However, at higher concentrations, A18 does decrease β-galactosidase activity in Δ-α 97Q cells while still increasing the β-galactosidase activity of Δ-α 23Q cells. Therefore, while there was no specificity at low concentrations, the specificity at high concentrations has the potential of making this a compound worth pursuing.

In Class III are compounds A25 and A31, which decreased the β-galactosidase activity in Δ-α 97Q but had no effect on Δ-α 23Q cells (**Fig 4**). These two compounds are interesting because they have the potential of having selectivity for expanded Htt but not normal Htt. This contrasts with earlier identified compounds that have effects on both expanded and normal Htt.

Effects of Class I, II, and III compounds on cell viability

Compounds that reduce cell viability would also cause a decrease in β-galactosidase activity in the assay. Therefore, the compounds were tested in a secondary assay for effects on viability. Although these compounds had been previously tested for toxicity effects, each cell line has different sensitivities to compounds. Therefore, they were tested for an effect on viability (MTS mitochondrial activity assay) of Δ-α 97Q and Δ-α 23Q cell lines. Cells were induced with PonA and either 5 or 10 μM compound for 24 hr, at which time the MTS solutions were added. 100% MTS activity was defined as the activity of induced cells without compound. A18 was the only compound that caused a decrease in viability in Δ-α 97Q cells (**Fig 5**). This specific decrease in viability could explain the decrease in β-galactosidase activity at 10 μM since a well with fewer healthy or proliferating cells would have lower β-galactosidase activity.

It is also interesting to note that no compound at 5 or 10 μM increased viability in Δ-α 23Q cells. This indicates that the increase in β-galactosidase activity observed in Δ-α 23Q cells with Classes I (**Fig 2**) and II (**Fig 3**) compounds was not due to an increase in cell number.

Effects of Class I, II, and III compounds on β-galactosidase and Htt protein levels

Since β-galactosidase activity level was being used as a reporter for Htt protein levels, it was important to verify the assay results by measuring protein levels. Immunofluorescence was used on Δ-α 97Q and Δ-α 23Q cell lines which had been induced and treated with 10 μM compounds for 24 hr. Of all seven specifically acting compounds, only A9 and A24 were capable of decreasing the level of α 97Q immunofluorescence (**Figs 6 and 7**). Comparison of induced Δ-α 97Q or Δ-α 23Q cells with DMSO vs. compounds showed that none of the

compounds had an effect on the cytoplasmic localization of Htt or β-galactosidase proteins (data not shown). In addition, none of the compounds had an effect on the level of β-galactosidase proteins in either cell line (data not shown).

Compound A9 at 10 μM very dramatically decreased the level of α 97Q proteins as compared to Δ-α 97Q cells which were treated with the drug vehicle DMSO (**Fig 6A and B**). However, it had no effect on either α 23Q or β-galactosidase Δ-subunit protein levels (**Fig 6C and D**). This indicates that A9 very specifically causes a decrease in expanded Htt protein levels, but does not affect normal Htt or other proteins induced from the same promoter.

Compound A24 at 10 μM showed a similar pattern in that it decreased α 97Q protein levels (**Fig 7A and B**), although not as strongly as A9. Of additional interest, A24 actually increased the level of α 23Q protein (**Fig 7C and D**). This change in protein levels directly parallels the effect of the compound on β-galactosidase activity: it decreased β-galactosidase activity in Δ-α 97Q and increased β-galactosidase activity in Δ-α 23Q.

Compound A9 may decrease expanded Htt protein production

It was evident by looking at the expression of Htt proteins in Δ-α 97Q and Δ-α 23Q cells with the addition of A9 or A24 that these compounds decreased the amount of α 97Q after 24 hr of treatment. This decrease in α 97Q protein levels could be due to a decrease in the rate of α 97Q protein production, an increase in the rate of α 97Q protein degradation, or a combination of both.

To address this question, the β-galactosidase activity in the first 12 hr of induction was assessed in Δ-α 97Q and Δ-α 23Q cells. Cells were induced and treated with 10 μM A9 or A24, and β-galactosidase activity was measured every 2 hr (**Fig 8**). Between 6-10 hr of expression, both Δ-α 97Q and Δ-α 23Q cells with A9 had lower β-galactosidase activity as compared to induced cells without compound. However, at 12 hr the β-galactosidase activity in Δ-α 97Q cells was still lower, while Δ-α 23Q cells had caught-up to the control cells. This suggests that while A9 may prevent the accumulation of α 97Q and α 23Q proteins at first, this effect is maintained for a longer period of time with α 97Q but not α 23Q proteins.

In contrast, A24 had no effect on the increase of β-galactosidase activity in the first 12 hr of induction. This could suggest that the decrease in α 97Q protein observed at 24 hr with A24

may be due to increased degradation of α 97Q. Similarly, the increase in α 23Q protein levels observed could be due to reduced degradation of α 23Q.

Effect of Class I, II, and III compounds on Htt^{Q103} PC12 cell toxicity

In order to determine if the compounds had the ability to rescue cellular toxicity due to expressed expanded Htt, they were tested in Htt^{Q103} PC12 cells. These cells have been shown to have 50% toxicity upon induction of the expanded Htt [74]. Cells were induced with either 1, 5, 10, or 25 μM compound for 72 hr. The ability of the compounds to rescue toxicity was assessed by the previously mentioned MTS assay.

All four compounds in Class I showed an ability to rescue toxicity in Htt^{Q103} cells (**Fig 9A**). More specifically, A7 only had partial rescue at 1 and 5 μM, and even caused additional toxicity at 25 μM. A8, A9 and A24 had complete rescue, in a dose-dependent manner. Since A9 and A24 were the only compounds that decreased α 97Q protein levels, Htt^{Q103} cells treated with 10 μM A9 or A24 were fixed and any changes in the GFP-tagged aggregates were examined. Cells with DMSO control mainly had multiple aggregates per cell, infrequently had either one or no aggregates, and never showed diffuse GFP (**Fig 9B**). Cells treated with A9 mainly had either one or no aggregates, some had diffuse GFP, and rarely were there multiple aggregates per cell. Cells treated with A24 mainly had a single aggregate, occasionally had multiple aggregates per cell, and sometimes had diffuse GFP. Cells which had one aggregate did not show a diffuse GFP signal, indicating that A9 and A24 are most likely not affecting the aggregation of Htt. The ability of the compounds to prevent multiple aggregates per cell is likely due to an overall decrease in expanded Htt protein levels. The fact that nucleation of expanded Htt requires a critical mass supports this theory since cells with low Htt protein levels would be unable to form aggregates and thus have a diffuse expression pattern.

The only concentration of A18 that could rescue toxicity of Htt^{Q103} cells was 1 μM (**Fig 9A**). Perplexingly, it was at low concentrations that A18 increased the β-galactosidase activity in both Δ-α 97Q and Δ-α 23Q cells (**Fig 3**). Therefore, in this case, a compound that increases the reporter for Htt protein levels can also rescue toxicity in a different cell line. Of the two compounds in Class III, only A31 was able to rescue toxicity in Htt^{Q103} cells (**Fig 9A**).

Characterization of four non-specific acting compounds:

Four compounds in Class IV non-specifically decrease β-galactosidase activity

As mentioned before, the 11 compounds that caused 0-75% β-galactosidase activity in Δ-α 97Q cells were tested in Δ-α 23Q cells for their ability to specifically affect expanded Htt. The seven compounds that were found to act specifically on Δ-α 97Q have already been discussed. The four compounds that were found to decrease β-galactosidase activity in both Δ-α 97Q and Δ-α 23Q were A14, A15, A20, and A29 in Class IV (**Fig 10**). Each of these compounds non-specifically decreased β-galactosidase activity to 25-50% at 10 μM.

Effect of Class IV compounds on cell viability

The four non-specific acting compounds were tested for an effect on cellular viability of Δ-α 97Q and Δ-α 23Q cell lines with the MTS assay, in order to identify compounds whose effect on β-galactosidase activity can be explained by a decrease in cell number. Cells were induced PonA and either 5 or 10 μM compound for 24 hr, when the MTS solutions are added.

Compound A14 caused a decrease in viability in both cell lines (**Fig 11**). In addition to this, it became apparent when looking at the wells that this compound caused a decrease in cell number (data not shown). This decrease in cell number could easily explain the decrease in β-galactosidase activity, therefore this compound was not further studied.

Effect of Class IV compounds on β-galactosidase and Htt protein levels

In order to verify that the change in β-galactosidase activity with compound correlated with a change in Htt protein levels, immunofluorescence was used on Δ-α 97Q and Δ-α 23Q cell lines. Cells were induced and treated with 10 μM compounds for 24 hr. Comparison of induced cells with DMSO control vs compounds shows than none of the compound had an effect on Htt or β-galactosidase protein cytoplasmic localization (data not shown). However, A15, A20 and A29 were capable of decreasing the level of α 97Q and α 23Q proteins (**Figs 12, 13 and 14**).

Compounds A15 and A20 were similarly capable of decreasing both α 97Q and α 23Q protein levels (**Figs 12 and 13**). However, these compounds also slightly decreased the β-galactosidase Δ-subunit protein levels in Δ-α 23Q cells. While this may indicate that the compounds were acting on all induced proteins, it is not necessarily the case since they did not affect the levels of β-galactosidase Δ-subunit protein levels in the Δ-α 97Q cell line. A29

decreased the levels of α 97Q and α 23Q proteins (**Fig 14**). The protein level of β-galactosidase Δ-subunit was not affected, indicating that the decrease in Htt proteins was not a global effect on induced proteins, but specific to Htt.

Effect of Class IV compounds on Htt^{Q103} PC12 cell toxicity

In order to determine if the compounds had the ability to rescue cellular toxicity due to expressed expanded Htt, they were tested in Htt^{Q103} PC12 cells, which have 50% toxicity upon induction of the expanded Htt. Cells were induced with either 1, 5, 10, or 25 μM compound for 72 hr. The ability of the compounds to rescue toxicity was assessed by assaying cellular viability with the MTS assay. Only compound A15 could partially rescue Htt^{Q103} toxicity (**Fig 15**). Compound A14 had the same toxic effect on Htt^{Q103} that it did on Δ-α 97Q and Δ-α 23Q cells, validating the decision to not study it any further.

DISCUSSION

In the attempts to find a treatment for HD, many cell models have been created to recapitulate some aspect of the disease. In many cases, the disease phenotype that was being targeted was toxicity [75, 76]. In other cases, inhibition of aggregation was the target [77]. While some of these assays have lead to the identification of interesting compounds, they each target a very specific aspect of the disease. In the hopes of finding a compound which would have the most dramatic effect on the disease, a cellular assay was designed to target the basic cause of HD: expression of the expanded Htt protein.

In order to identify compounds that target expanded Htt, a cellular assay was created which used β-galactosidase activity as a reporter for the presence or absence of expanded Htt or normal Htt. In these cells, Δ-α 97Q and Δ-α 23Q cell lines, the amount of α 97Q and α 23Q protein levels has been shown to directly correlate with β-galactosidase activity. Thus compounds that decrease β-galactosidase activity in Δ-α 97Q cells might also decrease the levels of expanded Htt.

To validate Δ-α 97Q cells as a useful drug-discovery assay, a collection of compounds previously shown to decrease the protein levels of a small Htt fragment and 103Q was tested. Since the Δ-α 97Q cell line contains the first 600 aa of Htt and all of the known caspase cleavage sites [78], they have the ability for the Htt fragment to be cleaved down to a smaller fragment which could be recognized by the compounds from the 103Q screen.

From the 114 compounds tested in an HTS-format, 34 were found to decrease β-galactosidase activity in Δ-α 97Q cells. Upon re-testing, 11 compounds were found to reliably and consistently decrease β-galactosidase activity. Of these, seven were found to act specifically decrease β-galactosidase activity in Δ-α 97Q cells but not Δ-α 23Q cells, and were subcategorized into three classes defined by their dose response characteristics (**Table 1**). Class I consisted of A7, A8, A9, and A24 which caused a decrease in β-galactosidase activity of Δ-α 97Q cells, but an increase in Δ-α 23Q cells. Interestingly, compounds A7, A8, and A9 belong to the same structural family of compounds. Class II consisted of only A18 which had the ability to increase β-galactosidase activity in both Δ-α 97Q and Δ-α 23Q at low

concentrations, but decrease β-galactosidase activity at higher concentrations. While the trend of effect of A18 was similar on both cell lines, there was a shift in the dose response curve, making the decrease in β-galactosidase activity selective at 10 μM. In Class III were A25 and A31 which decreased β-galactosidase activity in Δ-α 97Q cells but had no effect on Δ-α 23Q cells. Finally, four compounds non-specifically decreased β-galactosidase activity in both cell lines, and were categorized into Class IV.

To understand more clearly the mode of action for each of these 11 compounds, three additional assays were performed: cellular viability, Htt protein levels, and rescue of Htt^{Q103} toxicity (**Table 2**). By examining how a compound performed across these experiments, two distinct ways in which a compound may act to decrease β-galactosidase activity in Δ-α 97Q cells can be distinguished: decrease in Htt protein levels, and decrease in cell number.

Five compounds were found that decreased expanded Htt protein levels. Compounds A9 and A24 in Class I selectively decreased α 97Q protein levels, while compounds A15, A20, and A29 in Class IV decreased both α 97Q and α 23Q protein levels. In each case, when a compound had no effect on β-galactosidase activity in Δ-α 23Q cells, it also had no effect on α 23Q protein levels. Similarly, those compounds which decreased β-galactosidase activity in Δ-α 23Q also decreased α 23Q protein levels. Since these compounds had no effect on cell viability or β-galactosidase delta protein levels, the likely mechanism for decreasing β-galactosidase activity is the decrease in α 97Q or α 23Q protein levels. However, the specific mechanism underlying the decrease in protein levels remains to be elucidated.

Another mechanism for a decrease in β-galactosidase activity which can be readily attributed to compounds is a decrease in cell number. Compound A18 in Class II caused a decrease in cellular viability in Δ-α 97Q, while the non-specific compound A14 in Class IV caused a decrease in both Δ-α 97Q and Δ-α 23Q cells. The toxic effects of these compounds at the doses tested suggest that they should not be pursued further, except perhaps at still lower doses.

The mechanism of action for the four remaining compounds (A7, A8, A25 and A31) remains elusive. Since compounds A7, A8 in Class I and A31 in Class III have no detrimental effect on cellular viability and they rescue Htt^{Q103} toxicity to some degree, they remain

compounds worth pursuing. Since their effect on β-galactosidase activity is specific to the expanded Htt cell line, they are compounds which in some way specifically recognize expanded Htt but not normal Htt. Possible mechanisms include binding to expanded Htt to either change its conformation or physically interfering with the two β-galactosidase subunits to prevent alpha-complementation. Relocalization of expanded Htt to a different cellular compartment to prevent alpha-complementation has been ruled out by examining Δ-α 97Q and Δ-α 23Q cells.

Another potential mechanism for decreasing β-galactosidase activity is decreasing the β-galactosidase delta protein levels. A likely reason why no compounds were found with this mechanism is that these compounds were already selected for their ability to decrease a small fragment of Htt.

Of the compounds studied, A24 has the highest potential of being a worthy for drug development. This compound has all the characteristics that would be ideal in treating the basic cause of HD: decreasing disease-causing expanded Htt protein levels, increasing the protective normal Htt protein levels, not affecting cellular proliferation, and functional rescue of a toxic cell line. In the 34,000 compounds from the Chembridge library initially screened, this compound was found to be the sole representative of its kind. However, these results serve as proof of principle that compounds with the properties desired for HD therapy can be identified. To identify compounds with similar properties to A24 two routes may be taken. First, medicinal chemistry can be used to create new variations of the A24 structure which may have increased potency for causing reduction in expanded Htt levels. Second, HTS screening of additional chemical libraries may lead to the identification of compound scaffolds with similar modes of action but increased potency. Success in either of these endeavors could bring the approach to HD therapy outlined here significantly closer to practical implementation.

MATERIALS AND METHODS

Cell culture

Δ-α 97Q and Δ-α 23Q PC12 cells were grown at 37°C with 5% CO_2 in DME medium supplemented with 15% fetal bovine serum, 2 mM penicillin-streptomycin, 2 mM L-glutamine. The pVgRXR, pIND β-galactosidase Δ-subunit, and pIND/Hygro α 97Q or α 23Q constructs were maintained with 0.2 mg/ml Zeocin, 0.25 mg/ml Geneticin, and 0.1 mg/ml Hygromycin, respectively.

Htt^{Q103} PC12 cells were grown at 37°C with 10% CO_2 in DME medium supplemented with 25 mM HEPES, 5% calf serum, 5% horse serum, 2 mM penicillin-streptomycin, and 2 mM L-glutamine. The plasmid was maintained with 0.5 mg/ml Geneticin.

β-galactosidase activity assay

Δ-α polyQ cells were seeded at 50 x 10^4 cells/ml in 96-well plate format induced with 3 μM Ponasterone A (AG Scientific) in DMSO, and then grown at 37°C for 24 hr. Each well was then rinsed with PBS, and 10 μl of modified RIPA was added (150 mM NaCl, 50 mM Tris HCl pH 7.4, 1 mM EDTA, 1% NP-40, 1% w/v Na-deoxycholate, stored at 4°C). 67 μl of a master mix (5 μl 10x Cleavage Buffer, 0.135 μl 14.3 M β-mercaptoethanol, 44.865 μl dH_2O, 17 μl 4 mg/ml ONPG) from Invitrogen's β-galactosidase Assay Kit was added to each well and incubated at 37°C for 30-60 min. The addition of 125 μl of STOP Buffer (1 M Na_2CO_3) stabilizes the colorimetric change of ONPG cleaved by β-galactosidase, which was then read at 405 nm on a plate reader.

β-galactosidase activity is reported in nmoles of ONPG hydrolyzed. The absorbance at 405 nm can be converted to nmoles of ONPG hydrolyzed by the following formula:

$$\text{nmoles of ONPG hydrolyzed} = \frac{(\text{OD @ 405 nm}) * (\text{final vol} = 1.92 \times 10^5 \text{ nl})}{(4500 \text{ nl/nmole-cm}) * (1 \text{ cm})} = (OD_{405nm}) * (42.667 \text{ nmole})$$

The data from the experimental wells was normalized by subtracting the amount of β-galactosidase activity in un-induced wells. To report percent of β-galactosidase activity, 100% β-galactosidase activity was defined as the activity of induced cells, while 0% activity was

defined as the activity of un-induced cells. Subsequently, all experimental data is reported relative to these two controls.

High Throughput Screen

Δ-α 97Q cells were seeded on 96-well plates at 10 x 10^4 cells per well in 200 μl of media without selection drugs. In order to induce the expression of the β-galactosidase Δ and α 97Q constructs, PonA was added to a final concentration of 3 μM. Compounds from Chembridge were dissolved in DMSO and screened at 5-10 μM concentrations, in triplicate. Eight wells were used as positive controls and did not receive any compounds, and eight wells received un-induced cells to determine β-galactosidase background activity level. The screen was performed at MIND CAGn by Deb Russel and Steven Altman, supervised by Aleksey Kazantsev.

MTS assay

Δ-α polyQ cells were seeded at 10 x 10^4 cells/96-well or Htt^{Q103} PC12 cells at 2 x 10^4 cells/96-well and then grown for either 24 hr or 72 hr, respectively. 40 μl of MTS/PMS (Promega) was added to each well and incubated for 2-4 hr. The colorimetric change of MTS converted was read at 490 nm on a plate reader. The data from the experimental wells was normalized by subtracting the reading from medium-only wells.

Immunofluorescence

Δ-α polyQ cells were seeded at 50 x 10^4 cells/ml in chamber slides and grown for 24 hr. The cells were then fixed with 4% paraformaldehyde and blocked with 10% goat serum in PBS for 1 hr. MAB2166 Htt (1:500 Chemicon) or β-galactosidase (1:2000 MP Bio) antibodies and secondary antibodies (Alexa-488 and -594 1:200) were diluted in 10% goat serum and 0.2% Tween in PBS and incubated for 1 hr.

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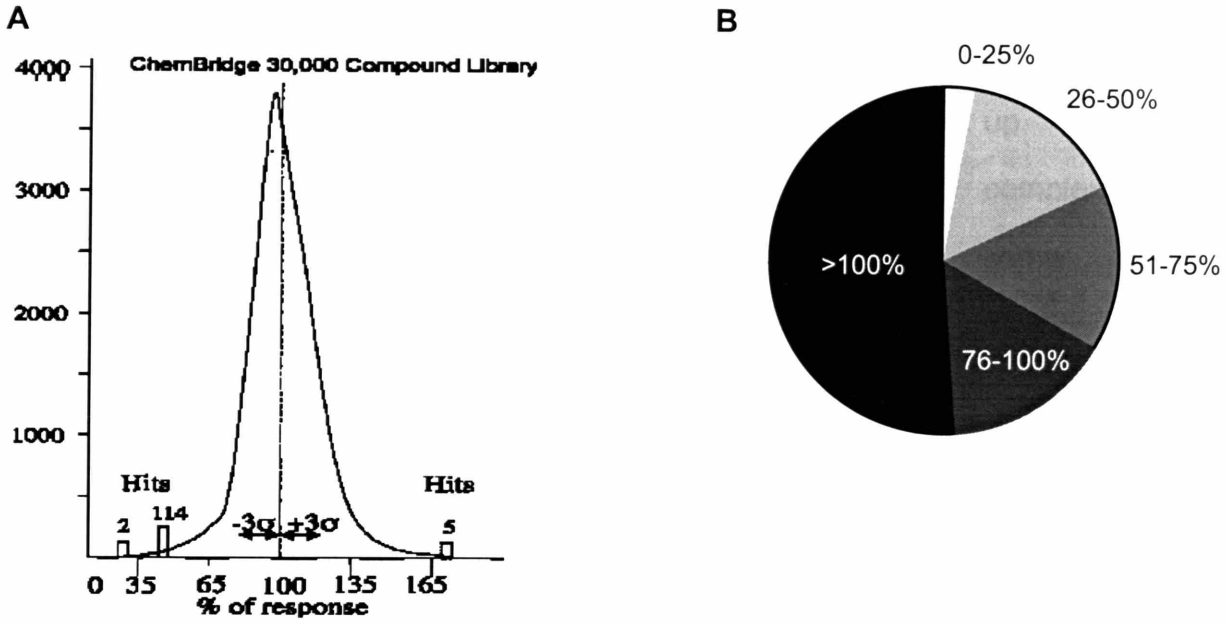

Figure 1: Verification of the compounds from HTS
A) Distribution of hits from 103Q HTS **B)** Distribution of hits from d-a97Q low-through put validation of 34 hits from d-a97Q HTS. d-a97Q cells were induced with 3 uM PonA and 10 uM compound for 24 hr. Beta-galactosidase activity was determined using Invitrogen's Beta-galactosidase Assay Kit. 100% Beta-galactosidase activity is defined as the activity of induced cells without compound, and the Beta-galactosidase activity of un-induced cells is subtracted from all data. Compounds were categorized into five groups: 0-25%, 26-50%, 51-75%, 76-100%, or >100% Beta-galactosidase activity. Of 34 compounds, 11 were found to reproducibly cause less than 75% Beta-galactosidase activity in d-a97Q cells and were further analyzed.

Class	Compounds	Relative Beta-galactosidase activity d-a97Q	d-a23Q
I	A7, A8, A9, A24	down	up
II	A18	complex	complex
III	A25, A31	down	same
IV	A14, A15, A20, A29	down	down

Table 1: Counter-screen d-a23Q categorizes 11 compounds into four classes
The 11 compounds verified to decrease Beta-galactosidase signal in d-a97Q were counter-screened in d-a23Q cells. Relative Beta-galactosidase activity is compared to induced cells without compound. Compounds in Class I raise the Beta-galactosidase signal in d-a23Q cells. The compound in Class II has complex effects in both cell lines. Compounds in Class III have no effect on Beta-galactosidase activity in d-a23Q cells. Compounds in Class IV decrease Beta-galactosidase activity in both cell lines.

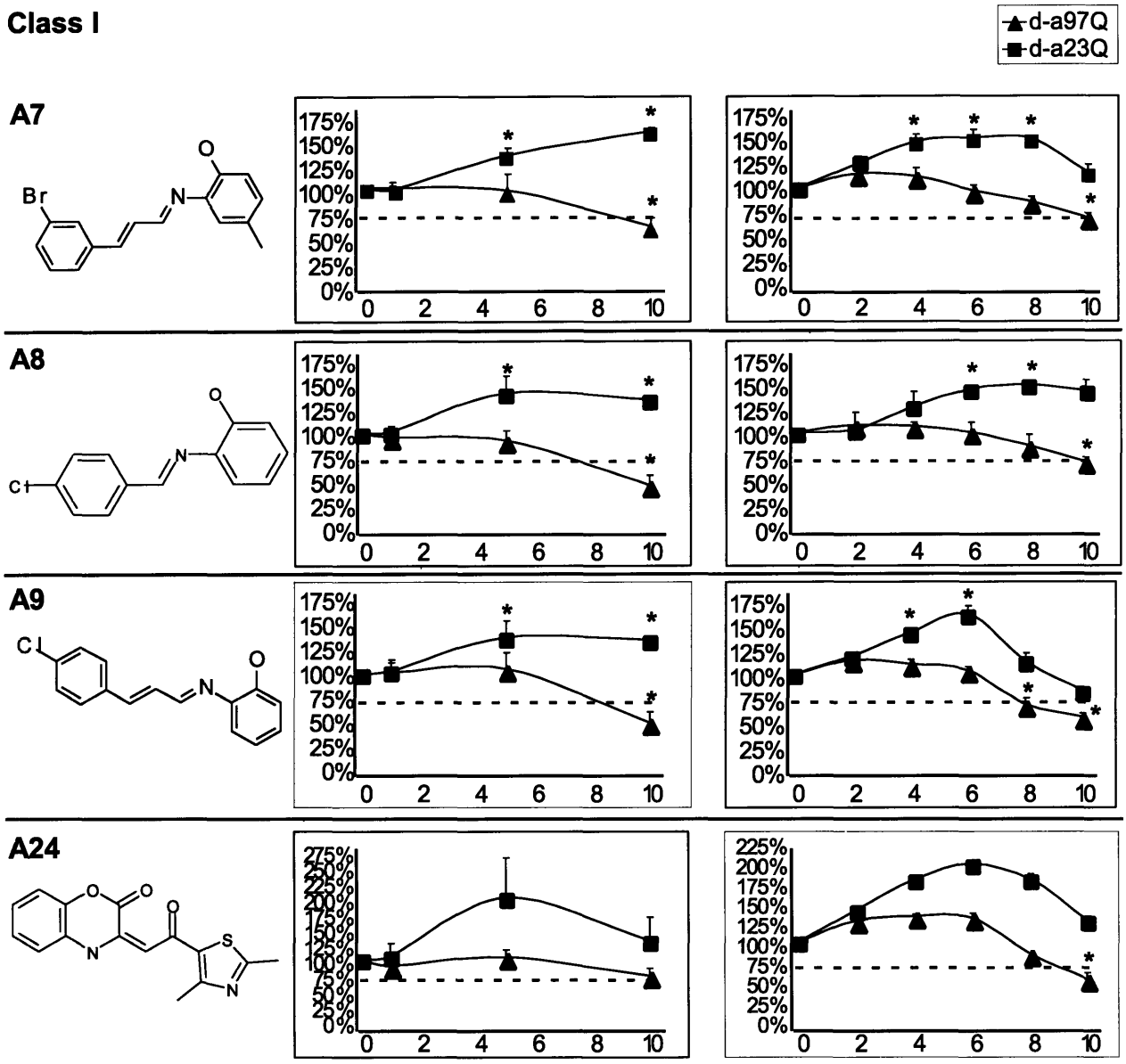

Figure 2: Class I compounds decrease Beta-galactosidase activity in d-a97Q but not d-a23Q

d-a97Q (triangles) and d-a23Q (squares) cells were induced with 3 uM PonA and upto 10 uM compound for 24 hr. Beta-galactosidase activity was determined using Invitrogen's Beta-galactosidase Assay Kit. 100% Beta-galactosidase activity is defined as the activity of induced cells without compound, and the Beta-galactosidase activity of un-induced cells is subtracted from all data. In each of these four compounds, Beta-galactosidase activity rises above 125% in d-a23Q but not in d-a97Q cells. Y-axis indicates relative Beta-galactosidase enzymatic activity. X-axis indicates uM compound. Error bars indicate standard deviation. Asterisk indicates $P<0.005$ when comparing vs. 0 uM. N=5 for data points in the first column of graphs, and N=3 for data points in the second column of graphs.

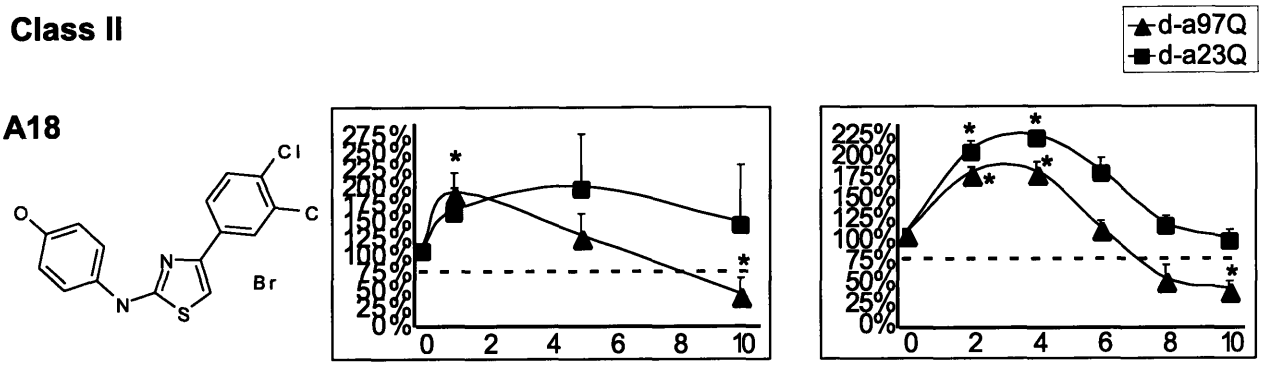

Figure 3: A18 decreases Beta-galactosidase activity in d-a97Q but not d-a23Q at high dosage

d-a97Q (diamonds) and d-a23Q (squares) cells were induced with 3 uM PonA and either 1, 2, 5, 8, or 10 uM compound for 24 hr. Beta-galactosidase activity was determined using Invitrogen's Beta-galactosidase Assay Kit. 100% Beta-galactosidase activity is defined as the activity of induced cells without compound, and the Beta-galactosidase activity of un-induced cells is subtracted from all data. With this compound, Beta-galactosidase activity rises above 125% in both d-a97Q and d-a23Q cells. Y-axis indicates relative Beta-galactosidase enzymatic activity. X-axis indicates μM compound. Error bars indicate standard deviation. Asterisk indicates $P<0.005$ when comparing vs. 0 uM. N=5 for data points in the first column of graphs, and N=3 for data points in the second column of graphs.

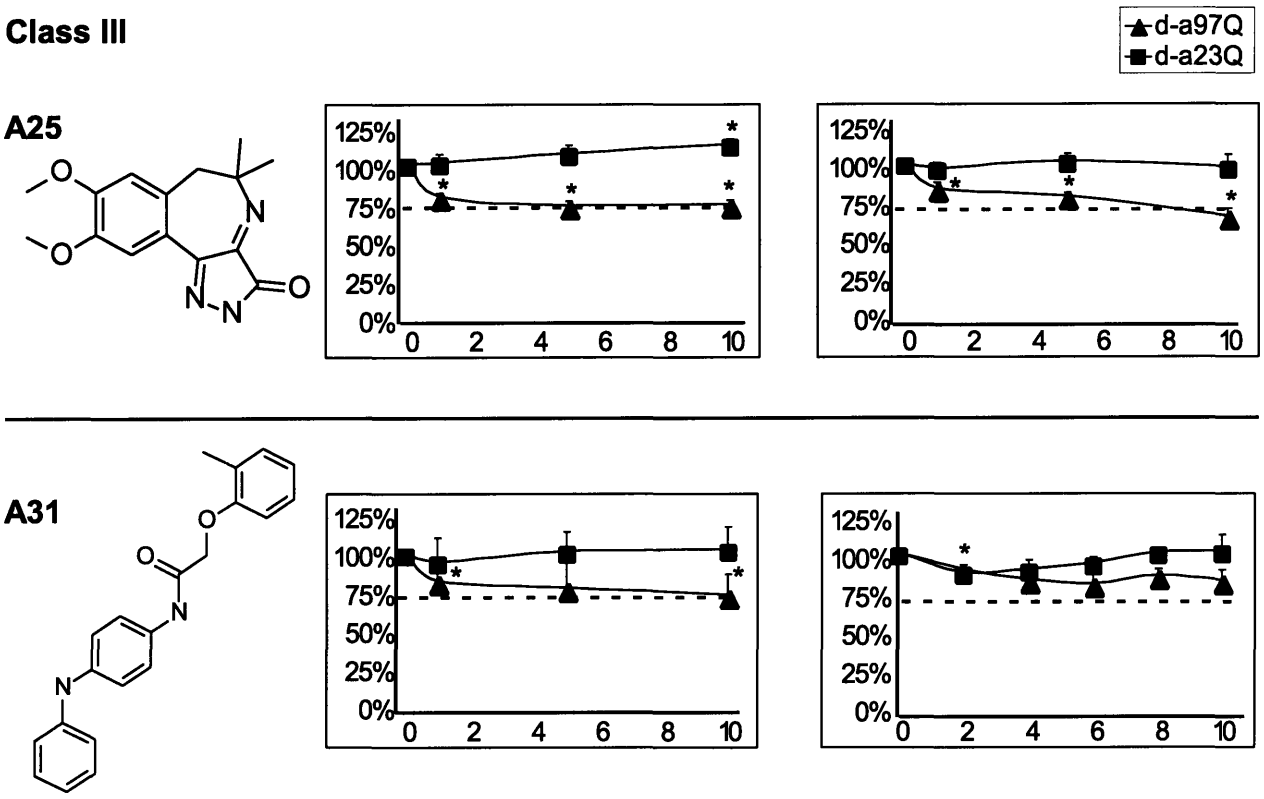

Figure 4: A25 and A31 decrease Beta-galactosidase activity in d-a97Q but not d-a23Q

d-a97Q (diamonds) and d-a23Q (squares) cells were induced with 3 uM PonA and either 1, 2, 5, 8, or 10 uM compound for 24 hr. Beta-galactosidase activity was determined using Invitrogen's Beta-galactosidase Assay Kit. 100% Beta-galactosidase activity is defined as the activity of induced cells without compound, and the Beta-galactosidase activity of un-induced cells is subtracted from all data. With these two compounds, Beta-galactosidase activity does not rise above 125% in either d-a97Q or d-a23Q cells. Y-axis indicates relative Beta-galactosidase enzymatic activity. X-axis indicates μM compound. Error bars indicate standard deviation Asterisk indicates $P<0.005$ when comparing vs. 0uM. N=5 for data points in the first column of graphs, and N=3 for data points in the second column of graphs.

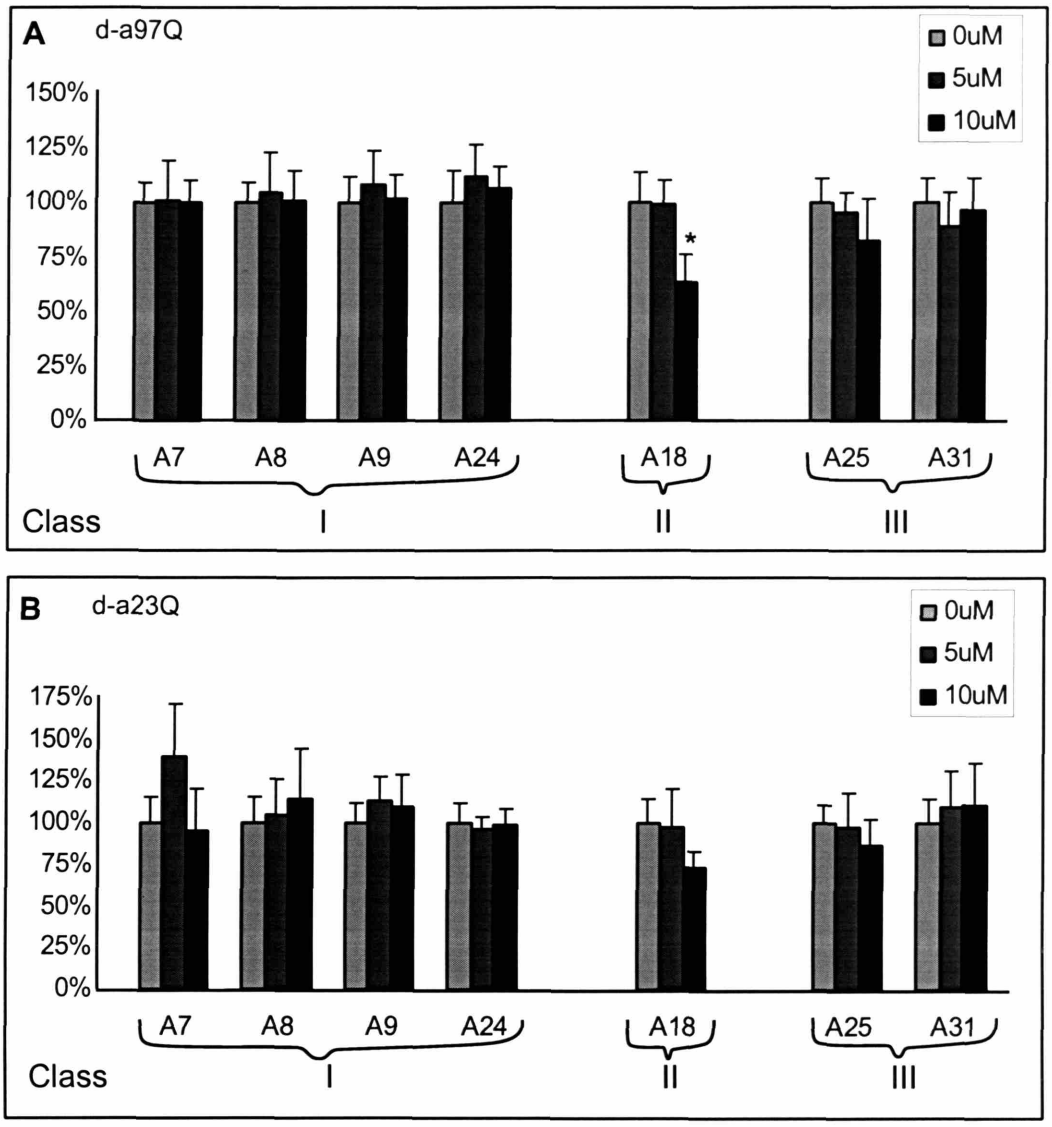

Figure 5: Only A18 decreases cellular viability
d-a97Q (**A**) and d-a23Q (**B**) cells were induced with 3uM PonA and either 5 or 10 uM compound for 24 hr. Cell viability was determined using Promega's MTS Assay Kit. 100% MTS activity is defined as the activity of induced cells without compound (0 uM). Y-axis indicates relative MTS activity. Error bars indicate standard deviation. Asterisks indicate $P<0.005$ when compared to 0 uM. N=6.

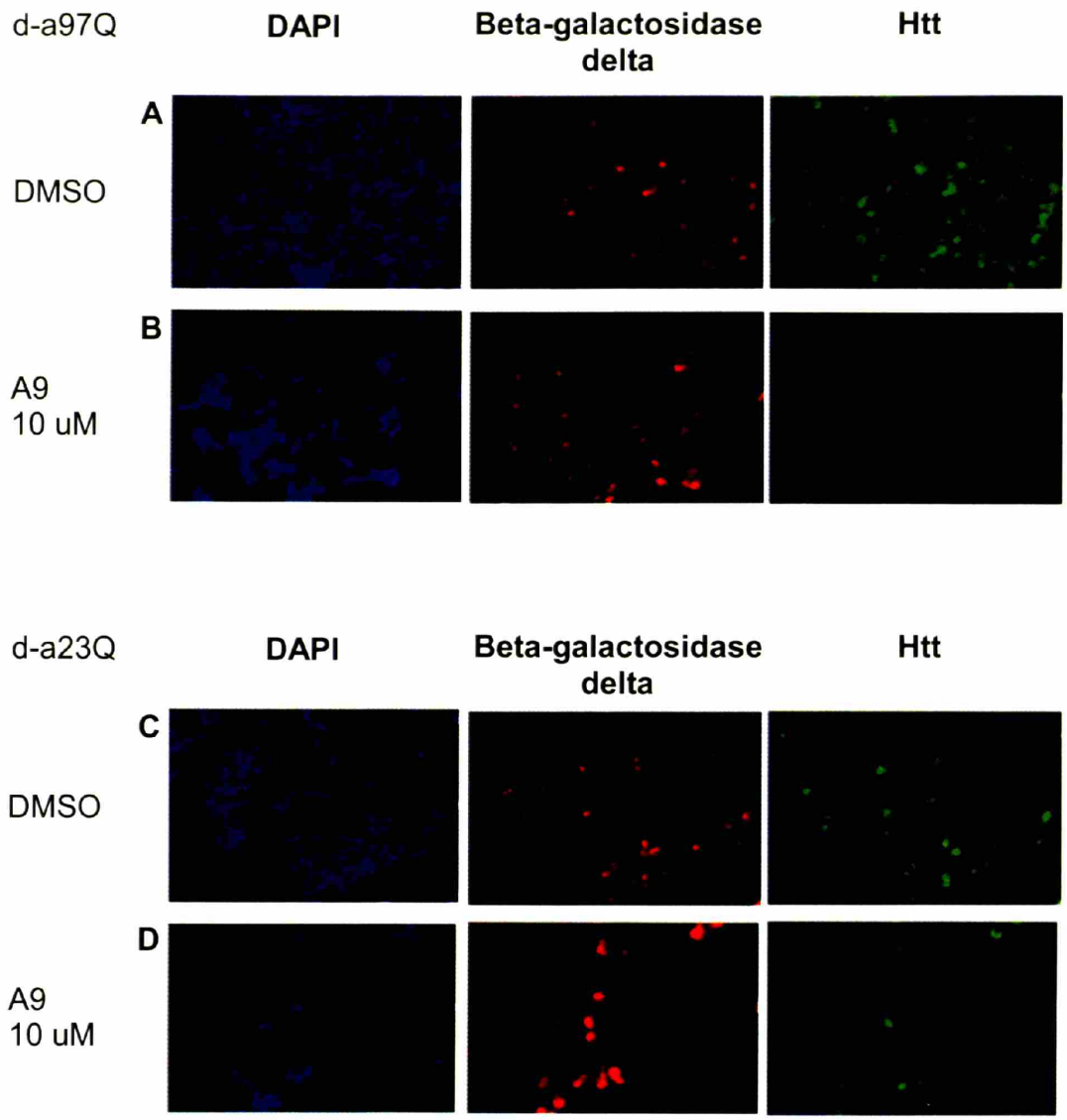

Figure 6: A9 decreases a97Q but not a23Q or Beta-galactosidase delta-subunit protein levels.
d-a97Q (**A-B**) and d-a23Q (**C-D**) cells were induced with 3 uM PonA and compound for 24 hr. DAPI (first column) stain was used to visual the nuclei, Alexa-594 for the Beta-galactosidase antibody (middle column), and Alexa-488 for the MAB2166 Htt antibody (last column).

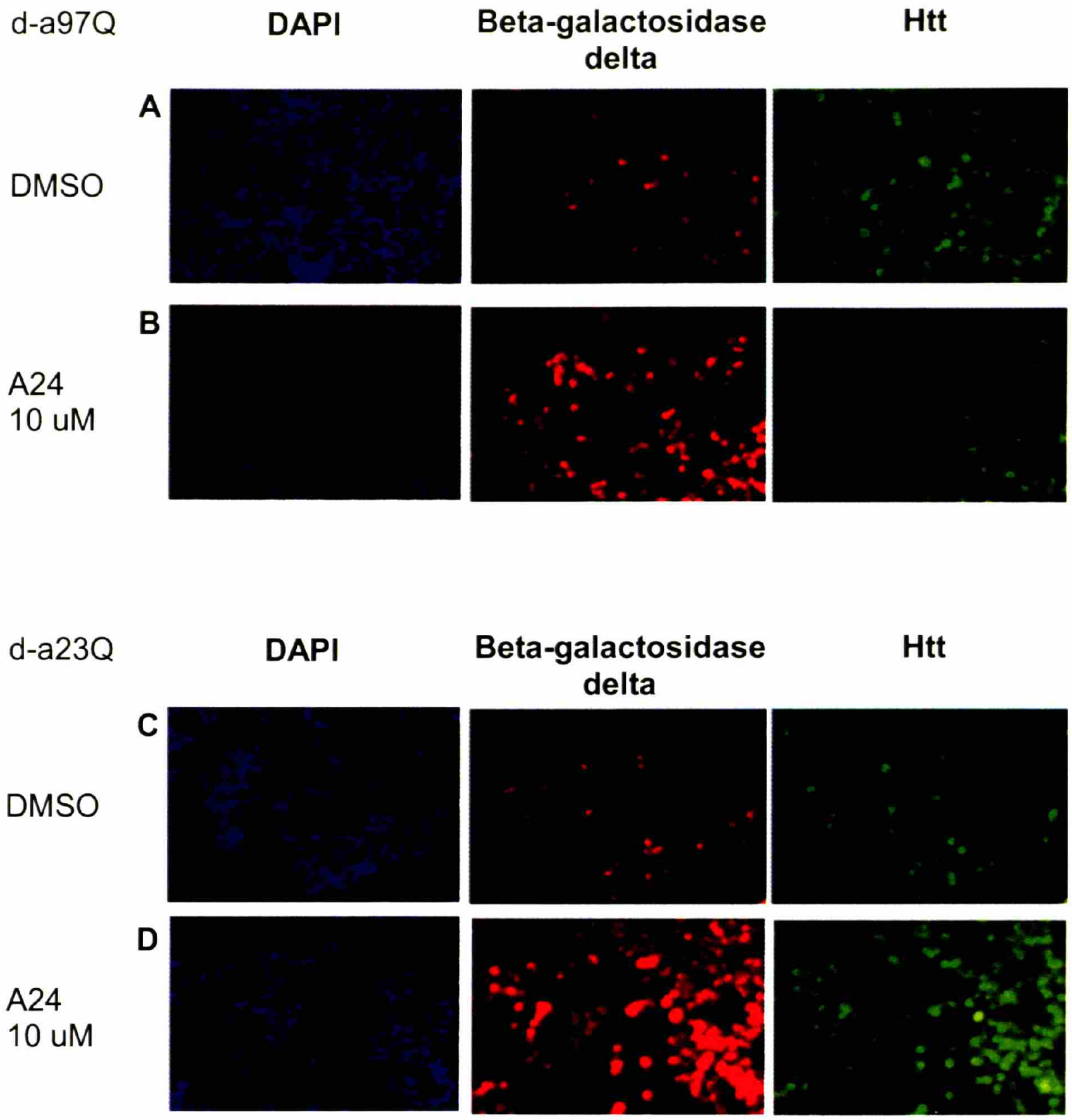

Figure 7: A24 decreases a97Q but increases a23Q protein levels.
d-a97Q (**A-B**) and d-a23Q (**C-D**) cells were induced with 3 uM PonA and compound for 24 hr. DAPI (first column) stain was used to visual the nuclei, Alexa-594 for the Beta-galactosidase antibody (middle column), and Alexa-488 for the MAB2166 Htt antibody (last column).

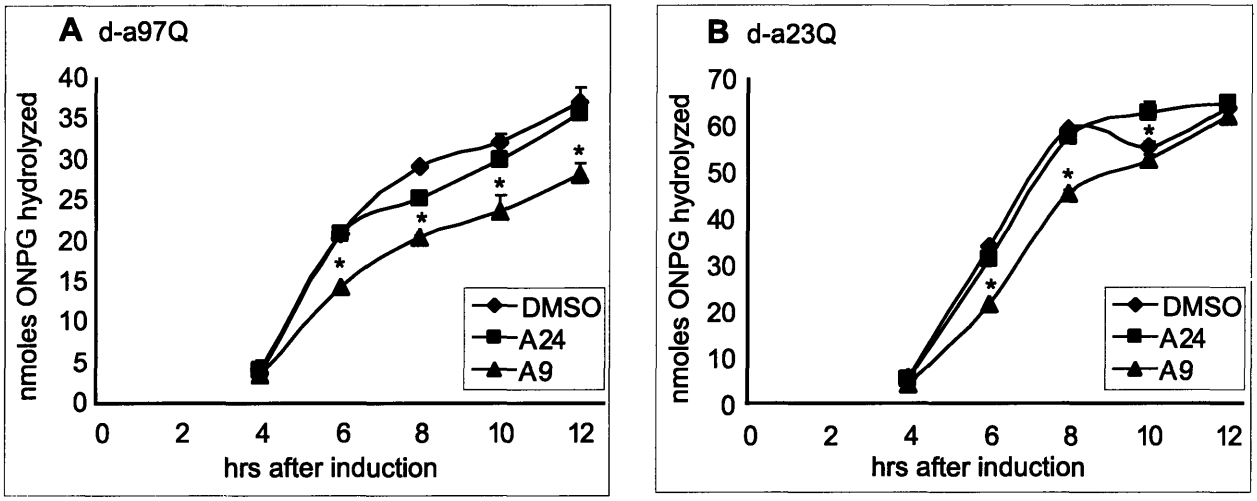

Figure 8: A9 lowers the rate of increase of Beta-galactosidase activity
d-a97Q (**A**) and d-a23Q (**B**) cells were induced with 3 uM PonA and either 10 uM of A24 or A9. Beta-galactosidase activity was assayed every 2 hr, beginning at 4 hr after induction and ending at 12 hr after induction. Error bars indicate standard deviation. Asterisks indicate $P<0.005$ when compared to induced cells without compound. N=4.

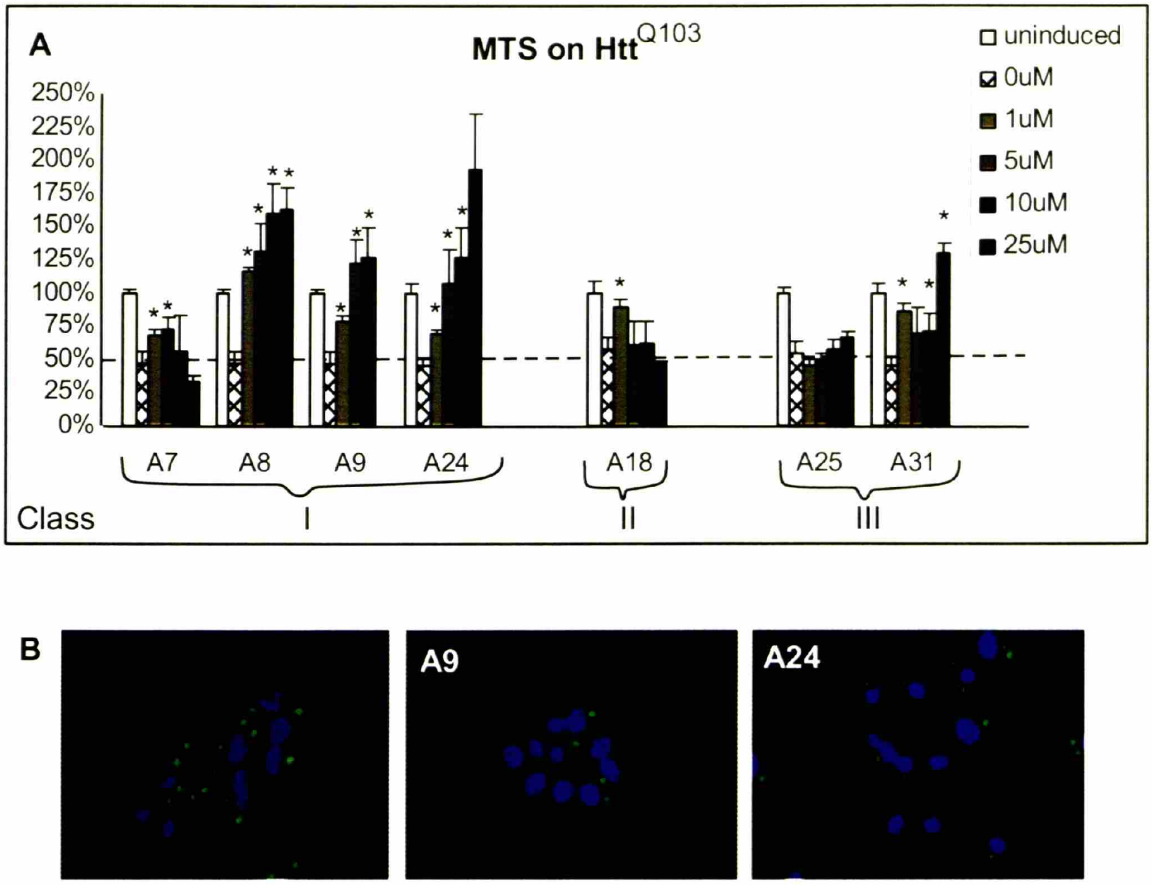

Figure 9: Almost all Class I and III compounds rescue toxicity of Htt^{Q103} PC12 cells, and compounds A9 and A24 alter the presentation of GFP aggregates

Htt^{Q103} PC12 cells were induced in the presence of either 1, 5, 10, 15, or 25 uM compound for 72hr. **A)** 100% MTS activity is defined as the activity of un-induced cells (white bars). A compound is determined to rescue toxicity when the MTS activity is significantly higher than that of induced cells with DMSO control (0 uM, hatched bars), emphasized with the dashed line. Y-axis indicates relative MTS activity. Error bars indicate standard deviation. Asterisks indicate $P<0.005$ when compared to induced cells without compound. N=6 for each data point, except the 25 uM data points where N=3. **B)** Comparison of induced cells at 72 hr with DMSO control or A9 or A24 at 10 uM. DAPI was used to stain nuclei, and green indicates the expression of the Htt^{Q103}-GFP construct.

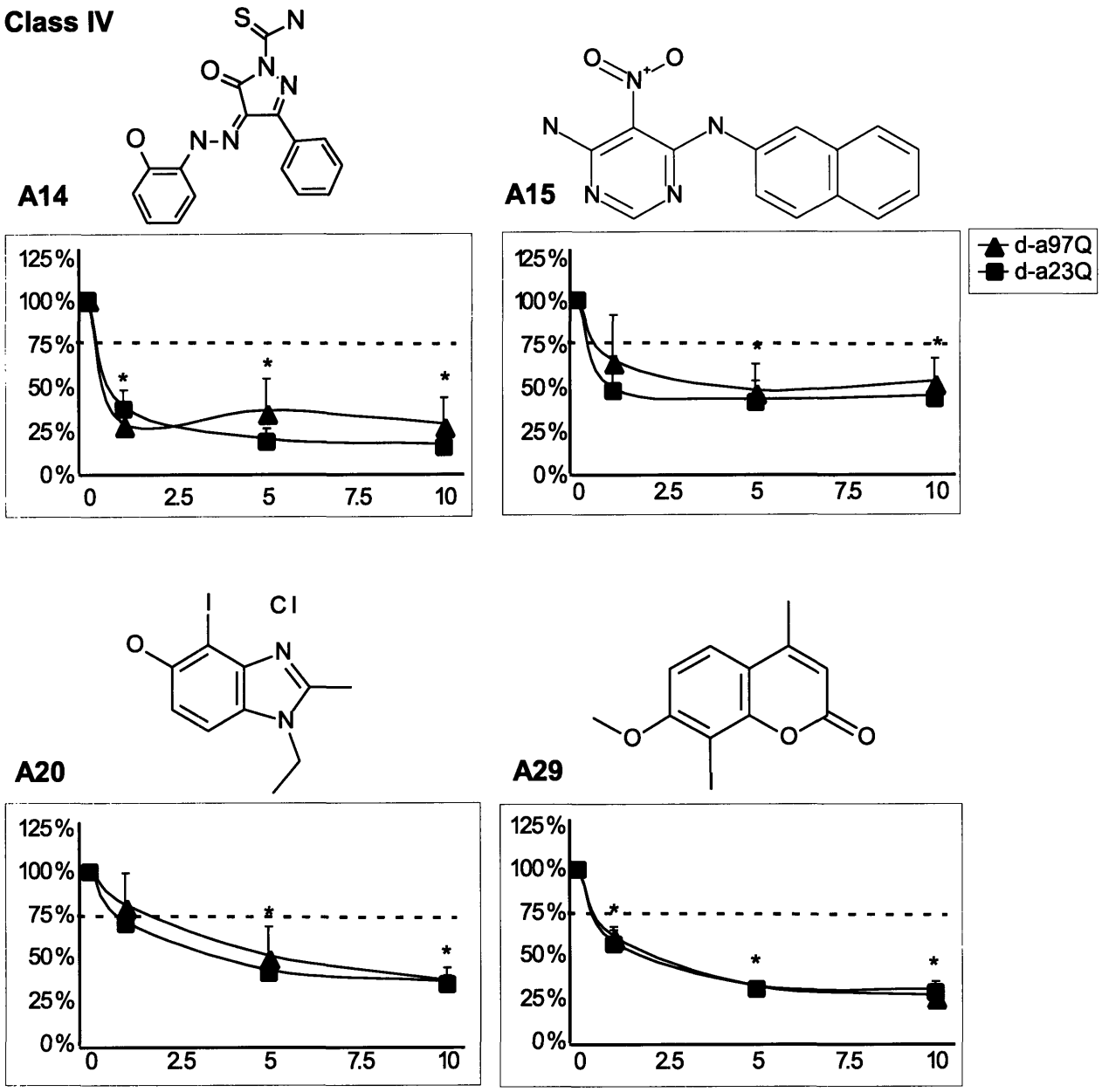

Figure 10: A14, A15, A20, and A29 decease Beta-galactosidase activity in both d-a97Q and d-a23Q.

d-a97Q (triangles) and d-a23Q (squares) cells were induced with 3 uM PonA and upto 10 uM compound for 24 hr. Beta-galactosidase activity was determined using Invitrogen's Beta-galactosidase Assay Kit. 100% Beta-galactosidase activity is defined as the activity of induced cells without compound, and the Beta-galactosidase activity of un-induced cells is subtracted from all data. Y-axis indicates relative Beta-galactosidase enzymatic activity. X-axis indicates uM compound. Error bars indicate standard deviation. Asterisks indicate $P<0.005$ when compared to induced cells without compound. N=5.

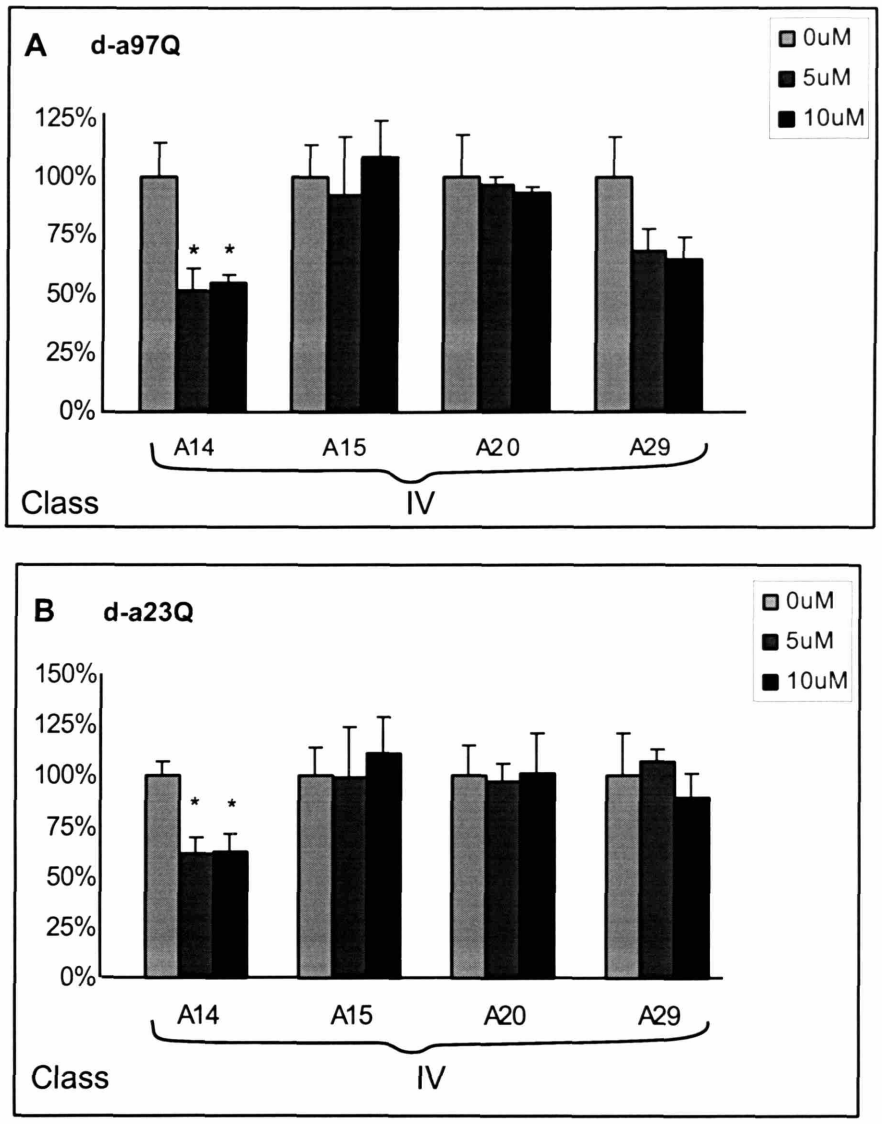

Figure 11: A14 decreases cellular viability
d-a97Q (**A**) and d-a23Q (**B**) cells were induced with 3 uM PonA and either 5 or 10 uM compound for 24 hr. Cellular viability was determined using the MTS Assay Kit. 100% MTS activity is defined as the activity of induced cells without compound. Y-axis indicates relative MTS activity. Error bars indicate standard deviation. Asterisks indicate $P<0.005$ when compared to induced cells without compound. N=6 for A14 and A15, N=3 for A20 and A29.

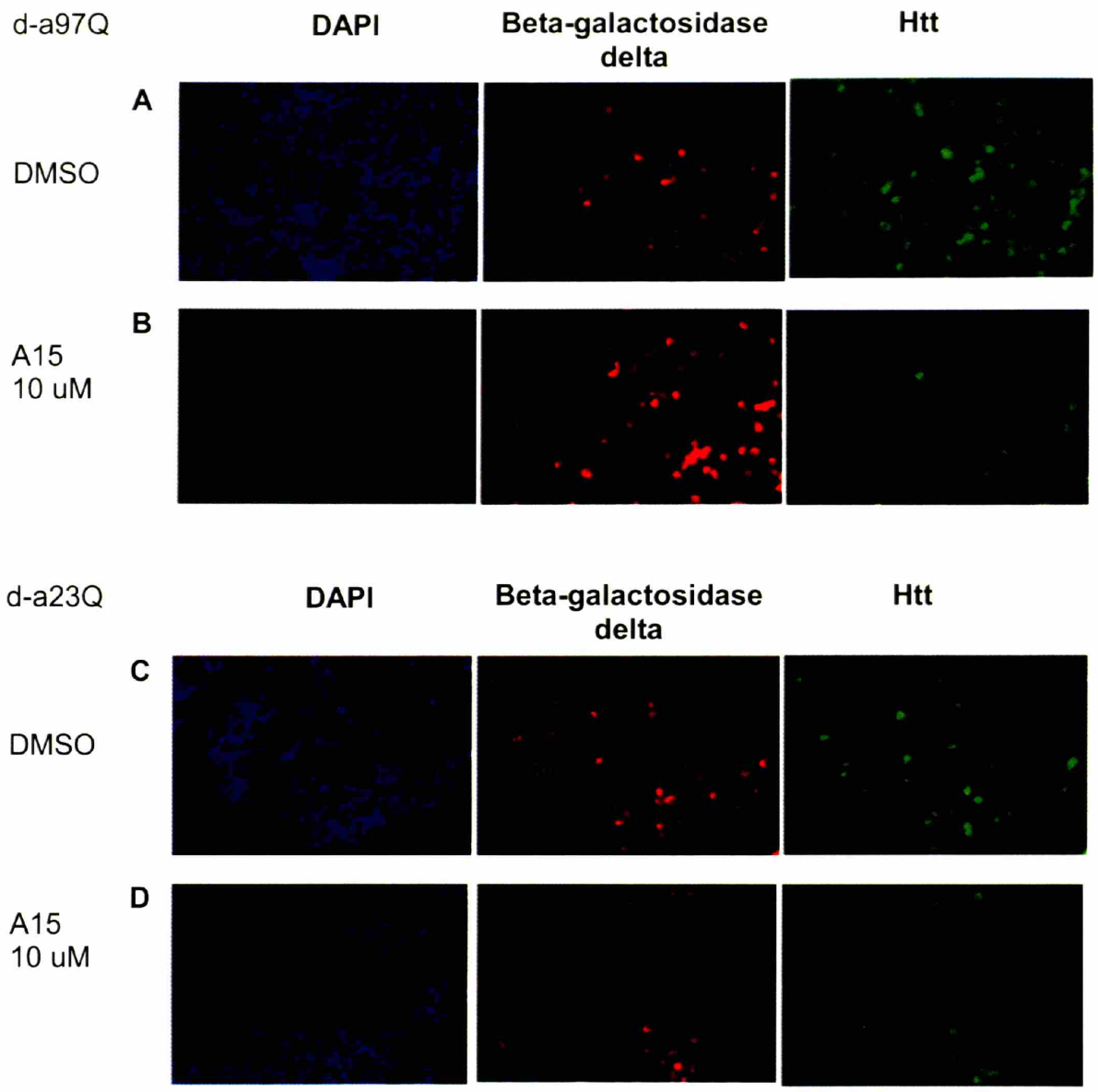

Figure 12: A15 decreases a97Q and a23Q protein levels
d-a97Q (**A-B**) and d-a23Q (**C-D**) cells were induced with 3 uM PonA and compound for 24 hr. DAPI (first column) stain was used to visual the nuclei, Alexa-594 for the Beta-galactosidase antibody (middle column), and Alexa-488 for the MAB2166 Htt antibody (last column).

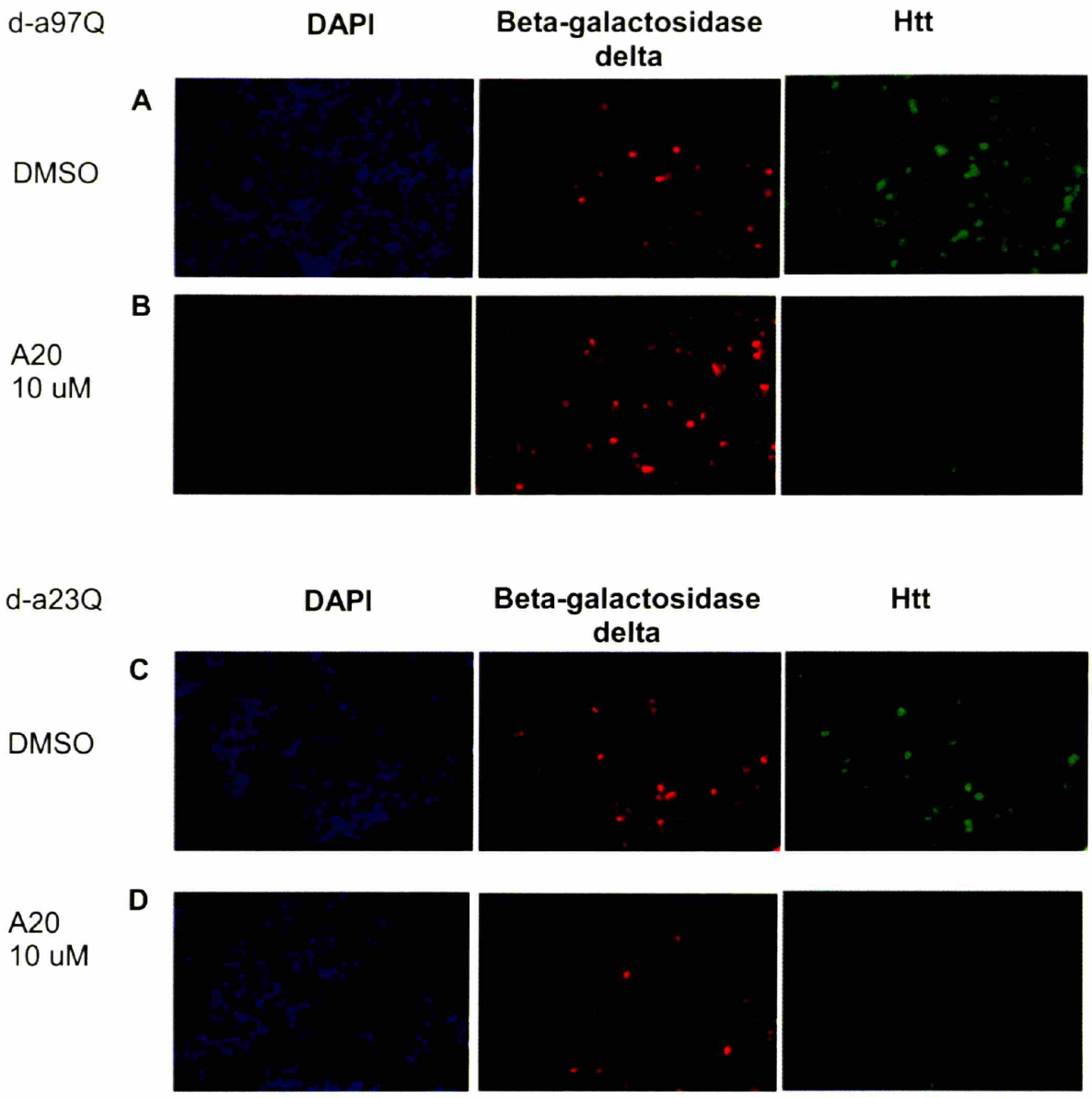

Figure 13: A20 decreases a97Q and a23Q protein levels
d-a97Q (**A-B**) and d-a23Q (**C-D**) cells were induced with 3 uM PonA and compound for 24 hr. DAPI (first column) stain was used to visual the nuclei, Alexa-594 for the Beta-galactosidase antibody (middle column), and Alexa-488 for the MAB2166 Htt antibody (last column).

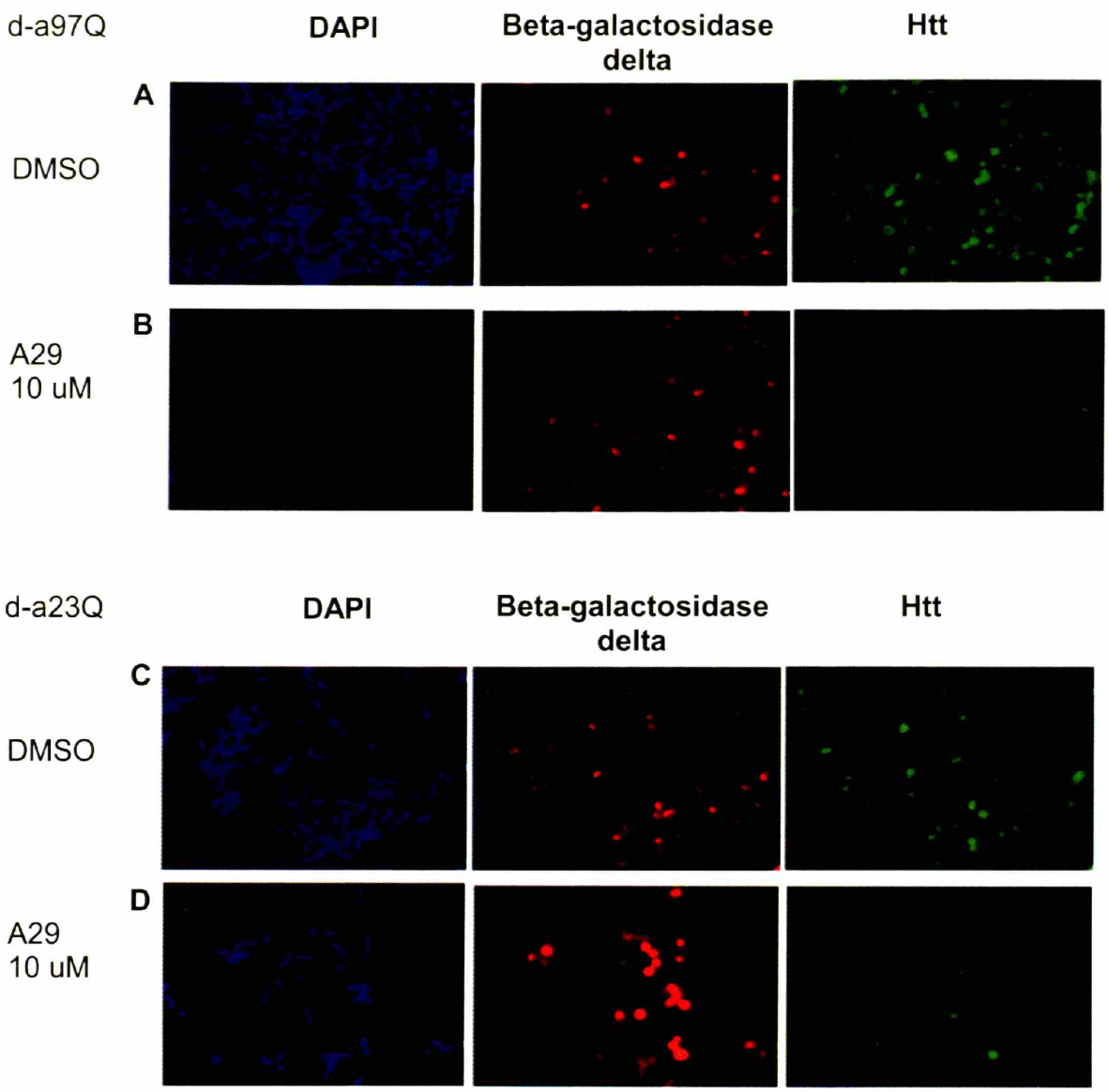

Figure 14: A29 decreases a97Q and a23Q protein levels
d-a97Q (**A-B**) and d-a23Q (**C-D**) cells were induced with 3 uM PonA and compound for 24 hr. DAPI (first column) stain was used to visual the nuclei, Alexa-594 for the Beta-galactosidase antibody (middle column), and Alexa-488 for the MAB2166 Htt antibody (last column).

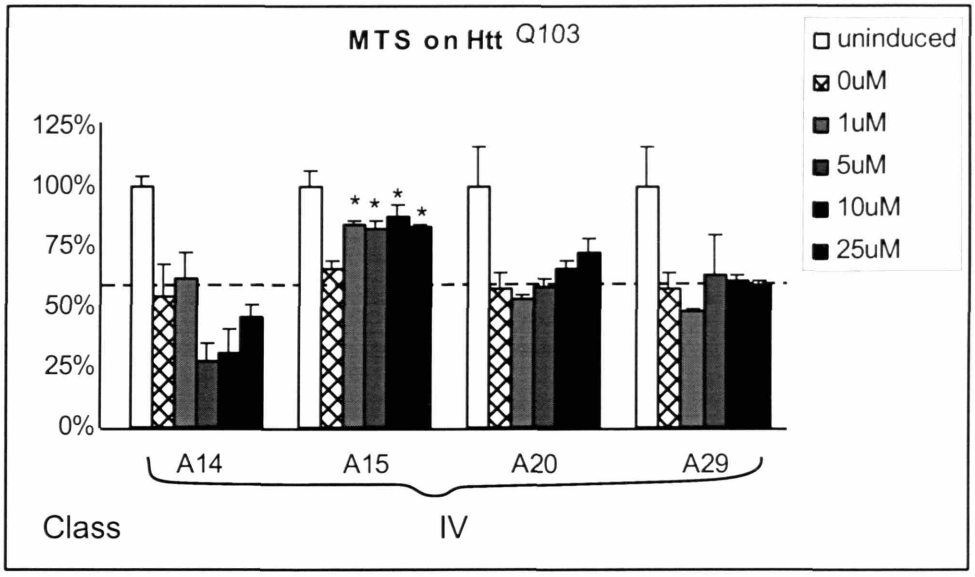

Figure 15: A15 rescues toxicity of Htt^{Q103} PC12 cells

Htt^{Q103} PC12 cells were induced in the presence of either 1, 5, 10, or 25 uM compound for 72 hr. 100% MTS activity is defined as the activity of un-induced cells (white bars). A compound is determined to rescue toxicity when the MTS activity is significantly higher than that of induced cells with DMSO control (0 uM, hatched bars), emphasized with the dashed line. Y-axis indicates relative MTS activity. Error bars indicate standard deviation. Asterisks indicate $P<0.01$ when compared to induced cells without compound. N=6, except A14 where N=6.

		Beta-galactosidase activity		cellular viability		protein levels		rescue Htt^{Q103} toxicity
		d-a97Q	d-a23Q	d-a97Q	d-a23Q	a97Q	a23Q	
I	A7	↓	↑	same	same	same	same	Yes
	A8	↓	↑	same	same	same	same	Yes
	A9	↓	↑	same	same	↓	same	Yes
	A24	↓	↑	same	same	↓	↑	Yes
II	A18	complex	complex	↓	same	same	same	No
III	A25	↓	same	same	same	same	same	No
	A31	↓	same	same	same	same	same	Yes
IV	A14	↓	↓	↓	↓	N/A	N/A	No
	A15	↓	↓	same	same	↓	↓	Yes
	A20	↓	↓	same	same	↓	↓	No
	A29	↓	↓	same	same	↓	↓	No

Table 2: Summary of the effects of each compound on assays tested
Table indicates summary of effect of each of the compounds on the assays tested: cellular viability of d-a97Q or d-a23Q, protein levels of a97Q or a23Q, and rescue of Htt^{Q103} toxicity. Results are relative to induced cells without compound, positive controls. The compounds are organized by Classes I, II, III, and IV. A9 and A24 have the most ideal characteristics across the assays, namely no affect on cellular viability, a decrease in a97Q but not a23Q protein levels, and rescue of Htt-^{Q103} PC12 toxicity.

CHAPTER IV

FUTURE DIRECTIONS EXTENDING THE WORK DESCRIBED HERE

ABSTRACT

A number of lines of investigation should be pursued to extend the work described here towards the ultimate goal of identifying an effective therapeutic intervention for HD. It would clearly be of significance to expand screening of chemical libraries in the hope of identifying compounds with higher levels of activity and alternative structures for further development through medicinal chemistry. An important line of investigation which should be pursued will be to establish the mechanistic basis by which identified compounds cause their effect on Htt protein levels. It would also be valuable to expand the range of cell and animal models in which compounds are characterized to develop a better understanding of the range of physiological conditions under which a compound will remain active in specifically decreasing endogenous expanded Htt protein levels. New cell lines can also be developed for more efficient screening and characterization of compounds. Finally, detailed analysis through medicinal chemistry to establish structure activity relationships for specific compound families would be an important part of this effort. In the course of this discussion some preliminary data that support the feasibility of particular lines of investigation is presented.

FUTURE DIRECTIONS

A cell-based assay was designed and constructed in order to identify compounds that specifically decrease expanded Htt but not normal Htt protein levels (**Chapter II**). β-galactosidase activity in these cells, Δ-α 97Q and Δ-α 23Q PC12 cells, was used as a reporter for the presence of expanded or normal Htt proteins. A collection of 114 compounds were screened in Δ-α 97Q cells in an HTS format successfully validating the assay (**Chapter III**). Of 34 compounds that emerged from the screen, 11 were confirmed by dose response curves in Δ-α 97Q cells. These 11 validated compounds were then counter screened in Δ-α 23Q cells to determine their specificity to expanded Htt. The compounds were further characterized by assessing their ability to decrease protein levels and functionally rescue the toxicity of another HD cell model. These data showed that the assay system described here utilizing Δ-α 97Q and Δ-α 23Q cells can be successfully used to identify compounds which specifically decrease expanded Htt protein levels.

In this chapter, I will discuss the future directions which I believe should follow these studies. In the course of this discussion I will present some preliminary data that support the feasibility of particular lines of investigation.

Direct use of the Δ-α 97Q and Δ-α 23Q cells as a primary HTS

It would clearly be desirable to identify additional compounds which have chemical and functional properties that are appropriate as a starting point for therapeutic intervention in HD. The compounds screened in Chapter III were pre-selected in that they were previously shown to decrease the levels of a smaller Htt fragment with 103Q. The use of Δ-α 97Q cells as a primary screening system would be preferable vs. a primary screen utilizing a smaller Htt fragment because the target polypeptide in the Δ-α 97Q screening system would be more likely to closely resemble the conformation of native full length Htt.

In order to assess the performance of Δ-α 97Q cells in a full-scale HTS, these cells were used to screen a 5,000 compound collection (from Maybridge) in collaboration with Aleksey Kazantsev and his laboratory. Thirteen compounds were found to decrease β-galactosidase

activity to below 75% of induced cells without compound. This hit rate of 0.26% was similar to other screens; for example, the 0.36% hit rate in the Htt-103Q PC12 cell line used to screen 36,000 compounds [1].

Dose response curves on Δ-α 97Q cells were used to verify the HTS results. At 10 μM, one compound had more than 75% β-galactosidase activity (**Fig 1**). This was considered a false positive and not further analyzed. The remaining 12 compounds showed linear dose responses, and at 10 μM had less than 75% β-galactosidase activity. Specifically, three had 0-25% β-galactosidase activity, three had 26-50% β-galactosidase activity, and six had 51-75% β-galactosidase activity.

The 12 compounds that caused 0-75% β-galactosidase activity in Δ-α 97Q cells were then tested in Δ-α 23Q cells for their ability to specifically affect expanded Htt. Three compounds did not decrease the β-galactosidase activity of Δ-α 23Q cells below 75% and were considered compounds with specificity to expanded Htt (**Fig 2**). The three specifically acting compounds (M4, M5, and M6) each caused no change in β-galactosidase activity in Δ-23Q cells, and thus were categorized into Class III of compounds previously described in Chapter III. While compounds M4 and M5 caused the most striking difference between β-galactosidase activities of Δ-α 97Q and Δ-α 23Q cells at 10 μM, compound M6 had the best effect at 1 μM.

Of the 12 compounds which caused 0-75% β-galactosidase activity in Δ-α 97Q cells, nine were found to also decrease β-galactosidase activity in Δ-α 23Q cells and were categorized into Class IV of compounds previously described in Chapter III (data not shown). Of these, three had 0-25% β-galactosidase activity, four had 26-50% β-galactosidase activity, and one had 51-75% β-galactosidase activity in both cell lines. One compound caused a greater decrease in β-galactosidase activity in Δ-α 23Q cells than in Δ-α 97Q cells.

Further experiments should be performed to fully characterize the 12 compounds. The viability of cells with addition of compound should be determined with the mitochondrial activity MTS assay to identify those compounds that decrease β-galactosidase activity by decreasing cell number (similar to Chapter III Figs 5 and 11). Immunofluorescence on these cells should be performed to identify what effects these compounds have on α 97Q, α 23Q, or β-galactosidase Δ-subunit protein levels or cellular localization (similar to Chapter III Figs 6, 7,

12-14). Finally, these compounds should be tested on Htt^{Q103} PC12 cells to check their ability to functionally rescue toxicity (similar to Chapter III Figs 9 and 15). The results of these experiments will allow the characterization of compounds that cause a decrease in α 97Q protein levels and can functionally rescue toxicity, and are therefore of potential interest for further development. The effective use of the Δ-α 97Q cell based assay in an HTS format suggests that the use of this assay for the screening of additional chemical libraries is likely to be productive.

Mechanistic analysis of decrease in expanded Htt protein level with compounds

Chapter III described a series of experiments that were performed to help determine the mode of action of seven compounds found to decrease β-galactosidase activity specifically in Δ-α 97Q cells, and four compounds found to decrease β-galactosidase activity non-specifically in Δ-α 97Q and Δ-α 23Q cells. The analysis of these experiments showed that five compounds caused a decrease in Htt protein levels, two compounds specifically in Δ-α 97Q cells and three in Δ-α 97Q and Δ-α 23Q cells. The mechanistic basis for the activity of each compound is an important question which should be explored further.

Decrease protein production vs. increased protein degradation

A question of interest which can be answered with greater clarity is the extent to which a compound acts at the time of synthesis of the polypeptide chain to influence its rate of production vs. the potential effect on the stability of a polypeptide chain increasing its rate of degradation. One experimental protocol which can give insight into this question would be to examine the rate of decrease of β-galactosidase activity and immunofluorescence in Δ-α 97Q and Δ-α 23Q cells with the addition of compound, a wash-out experiment. **Figure 3** shows preliminary data with 10 μM A25 where Δ-α 97Q and Δ-α 23Q cells were induced for 24 hr. The media from each well was removed and replaced with either media without inducer, or media without inducer but with A25. In this case, A25 had no effect on the rate of decrease of β-galactosidase activity upon removal of inducer. A complementary experiment which can provide additional information on the question of a compound's effect the production vs. degradation of expanded Htt protein is an induction time course (similar to Chapter III Fig 8). This could be followed, in principle, by a pulse labeling experiment. In this protocol, protein

production is quantified by incorporation of a labeled amino acid such as ^{35}S Methionine. By running protein samples taken over a time course on an acrylamide gel, a change in the instantaneous rate of production of α 97Q in the presence of a compound can be determined. Taken together these experiments can distinguish effects of a compound on the instantaneous rate of production of α 97Q and the subsequent rate of degradation of α 97Q.

Decrease in the instantaneous rate of synthesis of expanded Htt

The identification of a mechanism of action for compounds which reduce the instantaneous rate of synthesis of α 97Q will be quite challenging. It is worth noting, however, that characterization of nascent polypeptide chain interactions inside the ribosome during synthesis provide strong evidence for distinctive chaperone activity within the ribosome tunnel as a nascent chain is undergoing elongation [2]. The events which occur during the folding and processing of nascent chains could be the sites of specificity for the action of compounds which impact on the instantaneous rate of synthesis of α 97Q. Characterization of α 97Q and α 23Q behavior in systems designed to recapitulate these effects *in vitro* could lead to a mechanistic understanding of the basis of action of compounds which affect these processes.

Increase in the rate of degradation of expanded Htt

Several experiments could elucidate what aspects of degradation could be affected by a compound which causes an increased degradation rate for α 97Q. One mechanism for an increase in degradation could be proteasome targeting, which has been implicated in HD [3]. To address this possibility, a variation on the wash-experiment can be performed. Here, Δ-α 97Q and Δ-α 23Q cells would be induced for 24 hr at which time the media is replaced with new media containing either a proteasome inhibitor and compound, or a proteasome inhibitor alone. If the β-galactosidase activity in cells with proteasome inhibitor and compound decreases as compared to cells with proteasome inhibitor but no compound, then the pathway of degradation is proteasome-independent.

Chaperones play a role in the rate of degradation and have also been implicated in HD [4]. If a compound affects the effectiveness of a chaperone that participates in the folding of α 97Q, this could result in a higher percentage of misfolded α 97Q proteins which, would then be

targeted for degradation. *In vitro* assays of chaperone activity could be useful in determining the impact of compounds on these pathways.

Of interest in this regard is a recent report showing that 17-AAG, an analog of geldanamycin, both hsp-90 inhibitors, specifically decreases protein levels of the Androgen receptor with expanded polyQ but not normal polyQ, and in doing so ameliorates an SBMA mouse model of its disease symptoms [5]. It was of interest to determine if this compound would also have specificity to expanded Htt, since this is precisely the type of compound which Δ-α 97Q and Δ-α 23Q cells were designed to identify in an HTS. However, 17-AAG was unavailable at the time so geldanamycin was tested instead. Interestingly, geldanamycin caused a decrease in β-galactosidase activity in both Δ-α 97Q and Δ-α 23Q cells, even at very low concentrations (**Fig 4**). Analysis of viability by the MTS assay will determine if geldanamycin decreases β-galactosidase activity by decreasing cell number (similar to Chapter III Figs 5 and 11), a possibility since it is known to have some toxicity. If this result is negative, immunofluorescence on cells would determine if geldanamycin decreases both α 97Q and α 23Q protein levels (similar to Chapter III Figs 6, 7, 12-14). If geldanamycin does indeed decrease both α 97Q and α 23Q protein levels, it may suggest that 17-AAG does not recognize a protein by its expanded polyQ region alone, but perhaps by the polyQ region in context of the protein. This would explain why 17-AAG can have specificity to expanded AR but geldanamycin would recognize both expanded and normal Htt. In addition, the effects of 17-AAG would ideally be tested on Δ-α 97Q and Δ-α 23Q cells in order to make this conclusion.

Further validation of compounds in additional cell-based and animal assay systems

To further the overall goals of identifying an effective therapeutic intervention for HD, it would be desirable to continue validating compounds on other HD assay systems. One such system could be a novel cell-based assay designed to determine the range of polyQ lengths recognized by the compound. It would also be beneficial to show that the compound decreases endogenous full length expanded Htt protein levels in other HD model cells. Finally, testing the compounds on HD animal models for rescue of symptoms is an essential step before proceeding to human clinical trials.

Decrease β-galactosidase activity in Δ-α (intermediate polyQ) PC12 cells

Testing the effect of compounds on Δ-α 97Q and Δ-α 23Q cells allows the determination of the specificity of a compound for expanded Htt. Creating new cell lines with intermediate lengths of polyQ would help determine at what polyglutamine length the compounds are specific. Compounds tested only in cell lines with very high polyQ lengths may not be effective for treating HD because the majority of HD patients have 40-50 glutamines [6]. It would therefore be import to utilize cell lines expressing Htt with polyQ lengths similar to those seen in most HD patients to validate compounds identified in screens based on longer poly Q lengths.

Decrease full length expanded Htt protein levels in cell models

It would be beneficial to examine the effects of the compounds on cell lines that express both full length expanded and normal Htt in an endogenous manner. One such cells line is StHdh Q111/Q7 which is a straital cell line from a knock-in transgenic mouse where 111Q have been knocked-in to one of the Htt alleles [7]. Another useful cell line would be human lymphoblasts cells from HD patients.

Preliminary work has been done to optimize the analysis of Htt protein levels from StHdh Q111/Q7 (**Fig 5A**) and human lymphoblast cells (**Fig 5B, C**). The Western blots for the full length Htt proteins show that the expanded and normal Htt proteins can be separated and identified (**Fig 5A and B**). This allows for the possibility of identifying a compound which decreases the expanded Htt protein levels in any of these cells. In addition, a Western blot for a collection of human lymphoblasts shows that endogenous Htt with different lengths of polyQ can be cleaved into different length Htt fragments (**Fig 5C**). This is helpful because these Western blots could also identify compounds which may not have any effect on full length but would decrease Htt fragments levels.

While Western blots have the ability to identify compounds which affect the levels of full length or fragment Htt proteins, they are not very effective in determining if there is a change in full length Htt which exist in aggregates. In order to address this, filter trap assays could be performed. In this experiment, protein extracts are run through a membrane that allows soluble proteins to pass through but aggregated proteins remain trapped. The amount of protein on the

membrane can then be quantified to determine if the addition of compound to cells affects the levels of aggregated protein.

Rescue of symptoms and premature death in animal models

Using cell models is a good start in identifying compounds which could be drug candidates. To continue with more complex systems, certain animal models can be tested. For example, the compounds can be tested for rescue of toxicity in an HD yeast model [8, 9]. Similarly, they can be tested for rescue of toxicity in an HD *Drosophila* model [10, 11]. If a compound succeeds in rescuing these animals, then it can be tested in an HD mouse model for rescue of behavior, neuropathology, and death [12].

Identification of compounds of higher activity

While some compounds identified thus far may be potential candidates for further development, it would clearly be desirable to identify compounds which show desirable activity at a lower concentration range. The screening of additional libraries is clearly one route to such compounds. The exploration of novel compounds structurally related to ones found to be active would be of significance. In addition, structure activity relationships may provide insight into the molecular target of each compound series. Two complementary routes towards the acquisition of structurally related compounds for each active compound are available.

Pre-existing compound libraries

The compounds tested thus far have been acquired from sources which have already synthesized many additional compounds which include structurally related compounds for each compound tested. An initial strategy which may be taken is therefore to acquire a series of such structurally related compounds and test them in the assays described here. A preliminary picture of structure activity relationships may emerge from such studies.

Medicinal chemistry

Despite the availability of many such compounds from suppliers, as a study of this type proceeds, it will likely become necessary to synthesize *de novo* a series of compounds which test specific hypotheses or are likely to have higher biological activity. The further development of a compound series which shows good activity in cell-based assays may also include attention to

structural features of candidate molecules which improve bioavailability, serum half-life, and blood brain barrier penetration, while retaining the original specificity for reduction in levels of expanded Htt protein.

Final thoughts

The original motivation for the work described in this thesis was to contribute to the development of a therapeutic intervention for HD. Upon learning about all the various cellular dysfunctions that contribute to the disease symptoms, I thought targeting only one of these pathways would not necessarily have any significant effect on the HD patient. Therefore, I believed that the optimal way to treat HD was to target all of the cellular dysfunctions, and the simplest way to accomplish this would be to use one drug that targets the common underlying cause for all of the dysfunctions. I rationalized that people who do not express the expanded Htt protein do not exhibit HD symptoms, therefore if I could identify a compound that eliminates this disease-causing protein then it would be the optimal way to treat the disease. It is my hope that the results and discussion in this thesis have provided an incremental contribution towards the development of a therapy for HD.

MATERIALS AND METHODS

Cell culture

Δ-α 97Q and Δ-α 23Q PC12 cells were grown at 37°C with 5% CO_2 in DME medium supplemented with 15% fetal bovine serum, 2 mM penicillin-streptomycin, and 2 mM L-glutamine. The pVgRXR, pIND β-galactosidase Δ-subunit, and pIND/Hygro α 97Q or α 23Q constructs were maintained with 0.2 mg/ml Zeocin, 0.25 mg/ml Geneticin, and 0.1 mg/ml Hygromycin, respectively.

Lymphoblast cells were grown at 37°C with 5% CO_2 in RPMI medium supplemented with 15% fetal bovine serum and 2 mM penicillin-streptomycin.

StHdh (Q111/Q7) cells were grown 33°C with 5% CO_2 in DME medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 200 ug/ml Geneticin.

β-galactosidase activity assay

Δ-α polyQ cells were seeded at 50 x 10^4 cells/ml in 96-well plate format induced with 3 μM Ponasterone A (AG Scientific) in DMSO, and then grown at 37°C for 24 hr. Each well was then rinsed with PBS, and 10 μl of modified RIPA was added (150 mM NaCl, 50 mM Tris HCl pH 7.4, 1 mM EDTA, 1% NP-40, 1% w/v Na-deoxycholate, stored at 4°C). 67 μl of a master mix (5 μl 10x Cleavage Buffer, 0.135 μl 14.3 M β-mercaptoethanol, 44.865 μl dH_2O, 17 μl 4 mg/ml ONPG) from Invitrogen's β-galactosidase Assay Kit was added to each well and incubated at 37°C for 30-60 min. The addition of 125 μl of STOP Buffer (1 M Na_2CO_3) stabilizes the colorimetric change of ONPG cleaved by β-galactosidase, which was then read at 405 nm on a plate reader.

β-galactosidase activity is reported in nmoles of ONPG hydrolyzed. The absorbance at 405 nm can be converted to nmoles of ONPG hydrolyzed by the following formula:

$$\text{nmoles of ONPG hydrolyzed} = \frac{(\text{OD @ 405 nm}) * (\text{final vol} = 1.92 \times 10^5 \text{ nl})}{(4500 \text{ nl/nmole-cm}) * (1 \text{ cm})} = (OD_{405nm}) * (42.667 \text{ nmole})$$

The data from the experimental wells was normalized by subtracting the amount of β-galactosidase activity in un-induced wells. To report percent of β-galactosidase activity, 100% β-galactosidase activity was defined as the activity of induced cells, while 0% activity was

defined as the activity of un-induced cells. Subsequently, all experimental data is reported relative to these two controls.

High Throughput Screen

Δ-α 97Q cells were seeded on 96-well plates at 10 x 10^4 cells per well in 200 µl of media without selection drugs. In order to induce the expression of the β-galactosidase Δ and α 97Q constructs, PonA was added to a final concentration of 3 µM. 5,000 compounds from Maybridge were dissolved in DMSO and screened at 5-10 µM concentrations, in triplicate. Eight wells were used as positive controls and did not receive any compounds, and eight wells received un-induced cells to determine β-galactosidase background activity level. The screen was performed at MIND CAGn by Deb Russel and Steven Altman, supervised by Aleksey Kazantsev.

Western blots

StHdh (Q111/Q7) cells were seeded at 10 x 10^4 cells/ml in 12-well plates. Cells were lysed in StHdh lysis buffer (20 mM Tris pH7.2, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100) and Complete Protease Inhibitors (Roche), ice 30 min.

Lymphoblast cells were seeded at 10 x 10^4 cells/ml in 6-well plates. Cells were lysed with Lysis Buffer (50 mM Tris pH8, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40) and Complete Protease Inhibitors (Roche), ice 30 min.

Protein concentration was determined using Protein Assay and BSA standards (Bio Rad). 40-100 µg of protein in SDS loading buffer were loaded on an 8.5% low-bis acrylamide gel in a PROTEAN II system (Bio-Rad), with β-mercaptoethanol in the running buffer. Proteins were transferred to PVDF (Millipore) using 10% MeOH in transfer buffer (25 mM Tris, 190 mM glycine), at 25V 8 hr in 4°C.

PVDF blots were blocked in PBST with 0.5% milk for 1 hr. Actin (1:500, Sigma), MAB2166 Htt (1:2000, Chemicon), or PolyQ (1:2000) antibodies were diluted in PBST with 0.5% milk and incubated for 2 hr at room temperature or overnight at 4°C. HRP-conjugated secondary antibodies were diluted in PBST with 0.5% milk and incubated with blots for 30-45 min. Proteins were visualized with ECL Plus (Amersham Biosciences), and blots exposed to MR film (Kodak).

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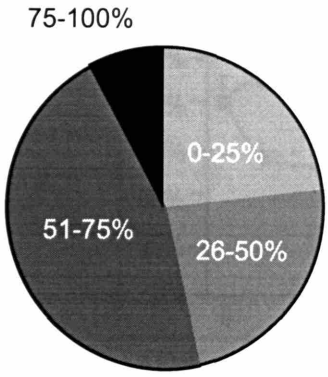
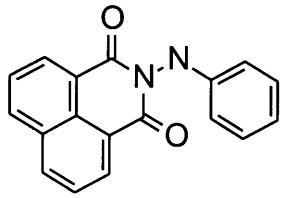
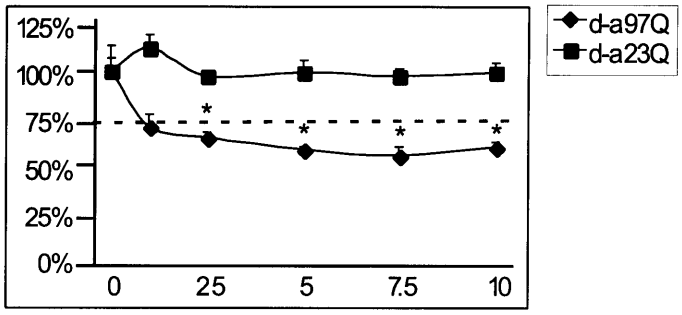


Figure 1: Distribution of 13 hits from HTS of 5,000 Maybridge compounds
d-a97Q cells were induced and treated with 10 uM compound for 24 hr. Compounds were categorized by their ability to decrease Beta-galactosidase activity. Categories are 0-25%, 26-50%, 51-75%, and 75-100% Beta-galactosidase activity as compared to induced cells without compound.

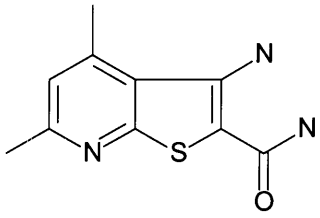
Class III

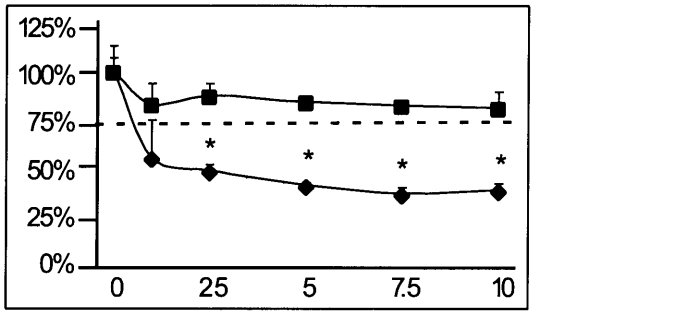
M4



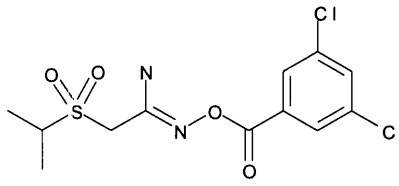


M5





M6



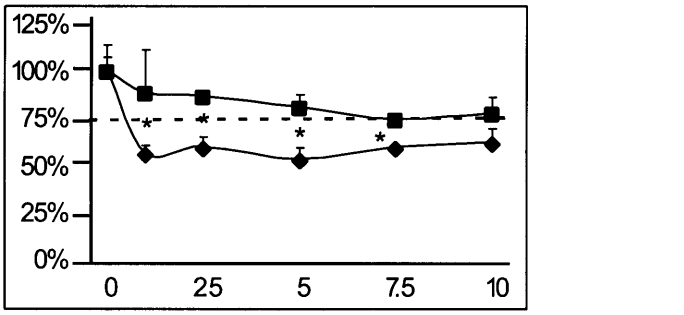


Figure 2: Dose response curves for 3 Maybridge specific acting compounds
d-a97Q (diamonds) and d-a23Q (squares) cells were induced and treated with either 1, 2.5, 5, 7.5, or 10 uM compound for 24 hr. Y-axis indicates relative Beta-galactosidase activity. X-axis indicates uM of compound. Error bars indicate standard deviation. Asterisks indicate $P<0.005$ when compared to induced cells without compound. N=2.

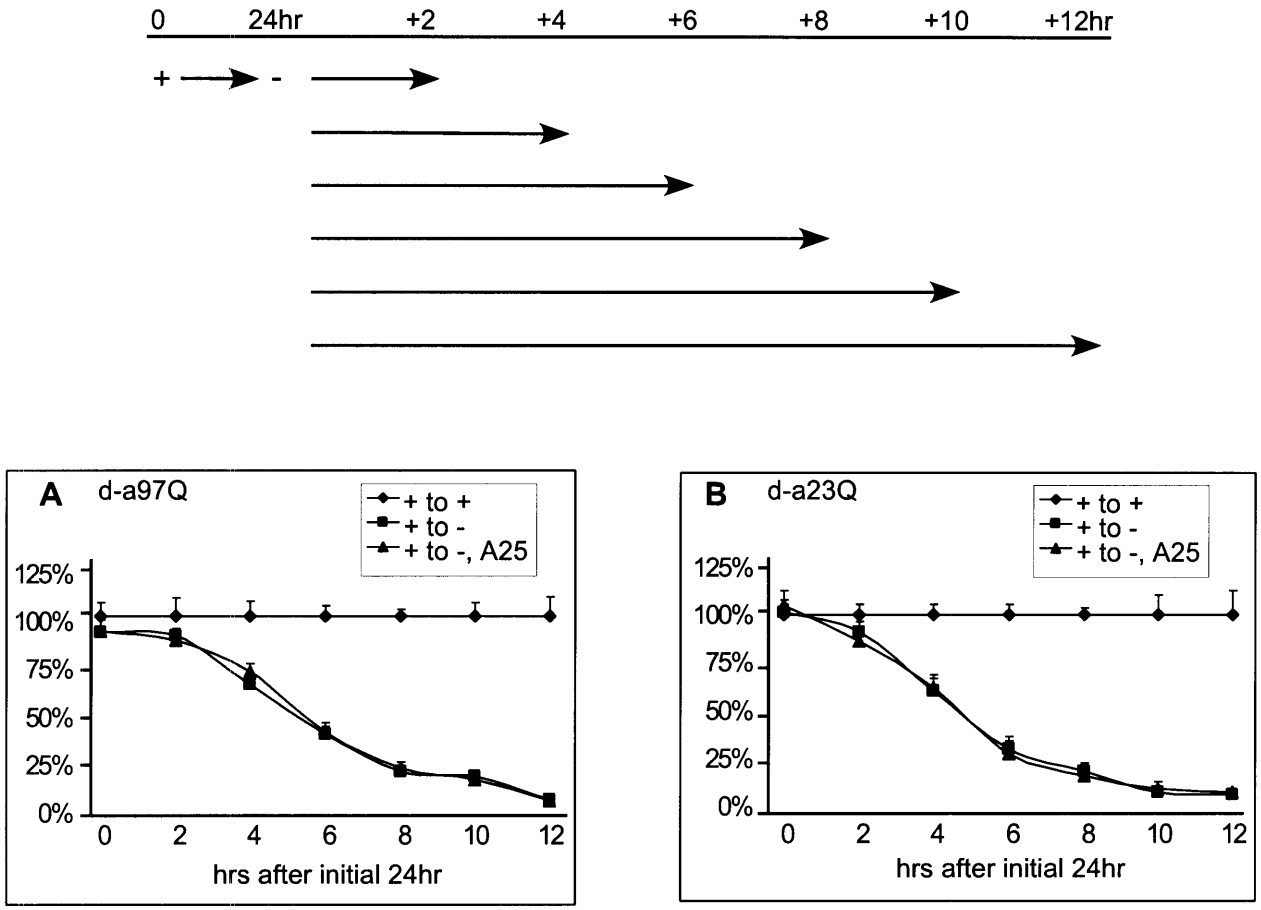


Figure 3: Sample Wash-out experiment with A25
d-a97Q (**A**) and d-a23Q (**B**) cells were induced for 24 hr. The media from each well was removed and replaced with either media with PonA (diamonds), media without PonA (squares), or media without inducer but with 10 uM A25 (triangles). Beta-galactosidase activity was assayed every 2 hr for 12 hr. Y-axis indicates relative Beta-galactosidase activity. Error bars indicate standard deviation. N=5.

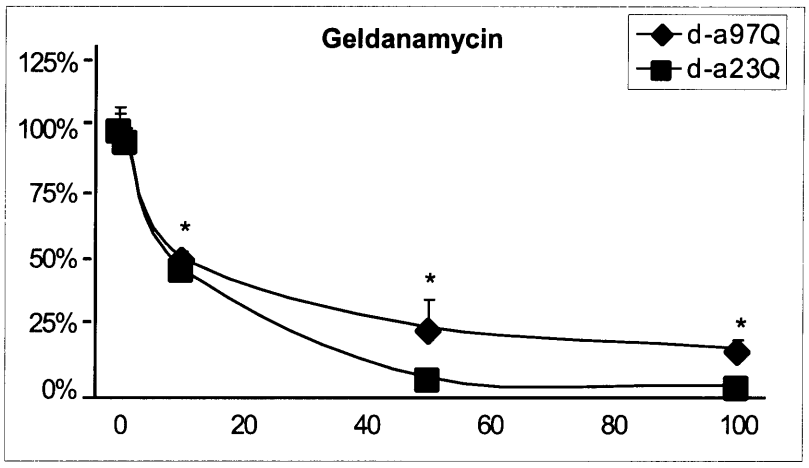


Figure 4: Geldanamycin decreases Beta-galactosidase activity in both d-a97Q and d-a23Q cells
d-a97Q (diamonds) and d-a23Q (squares) cells were induced and treated with either 1, 10, 50, or 100 nM geldanamycin. Y-axis indicates relative Beta-galactosidase activity. X-axis indicates nM of geldanamycin. Error bars indicate standard deviation. Asterisks indicate $P<0.01$ when compared to induced cells without compound. N=4.

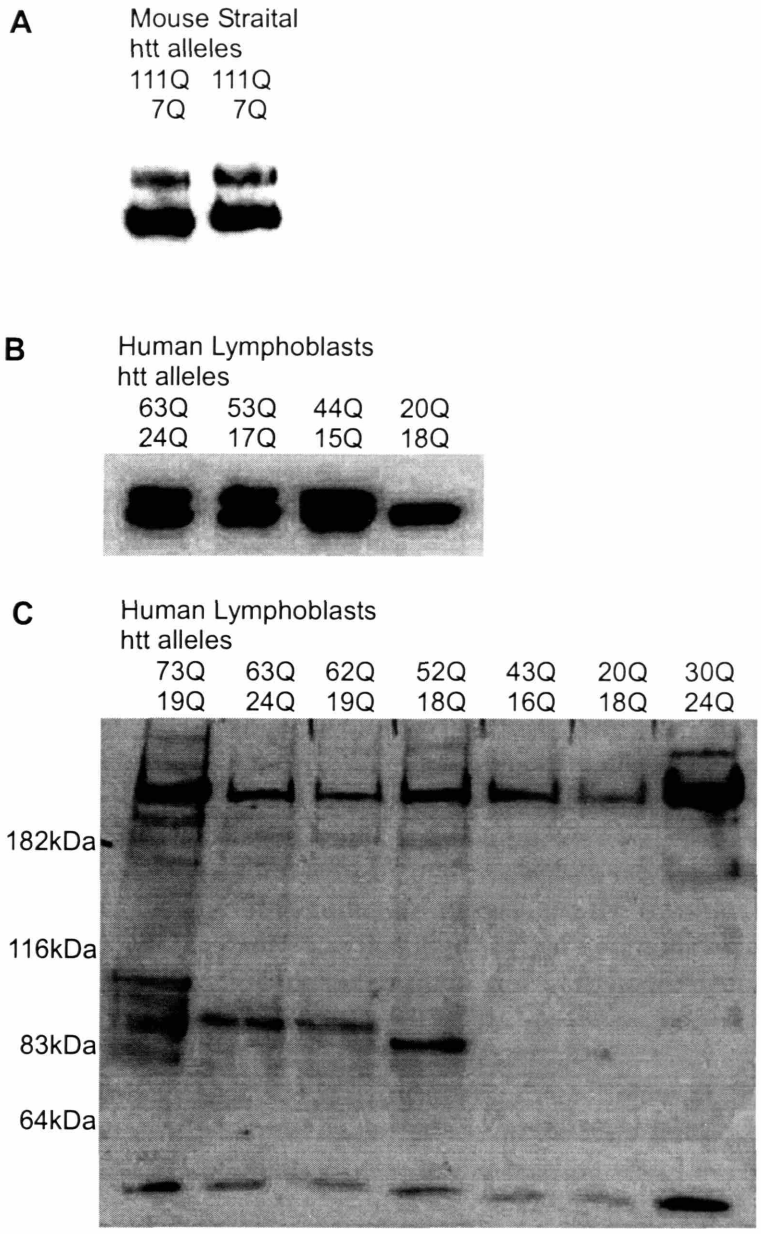


Figure 5: Sample Western blots of mouse straital and human lymphoblast cells
Lysates from StHdh Q7/Q111 (**A**) or various human lymphoblasts (**B, C**) were run on acrylimide gels and transferred to PVDF blots. A) PolyQ antibody recognized full length proteins. **B**) MAB2166 Htt antibody recognized full length htt proteins. **C**) PolyQ antibody recognized multiple Htt fragments.

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BIOGRAPHICAL NOTE

Education

Harvard University; Cambridge, MA 1995-1999
Cum Laude Biochemical Sciences AB thesis with Dr. William Gelbart:
Exploration of the Function of a New Activin-like Gene in *Drosophila Melanogaster*

Research Experience

Massachusetts Institute of Technology; Cambridge, MA 2002-2006
Dr. David Housman

- Developed cell-based assay for specific degradation of the mutant huntingtin protein to be used in 40,000 compounds High Throughput Screen, recently acquired by Novartis Int.
- Characterized compounds which emerged from High Throughput Screen
- Performed and analyzed behavioral tests on Huntington's Disease mouse model

Dana Farber Cancer Institute; Boston, MA 1999-2001
Drs. Lynda Chin and Ronald DePinho

- Published results of tumorigenesis study in a Melanoma mouse model
- Designed and implemented genotyping strategies for several investigators

Leadership and Teaching Experience

Massachusetts Institute of Technology; Cambridge, MA Spring 2005
Drs. Mary Lou Pardue and Frank Gertler

- Set-up a teaching laboratory by ordering supplies, testing equipment, modifying protocols, preparing starting materials, and creating a list of laboratory rules and jobs
- Supervised 19 undergraduate students develop and pursue a cell and molecular biology project by guiding their research
- Earned excellent student evaluations

Massachusetts Institute of Technology; Cambridge, MA Fall 2002
Drs. Eric Lander and Robert Weinberg

- Prepared and taught four supplementary classes a week and one review session for an undergraduate introductory biology course
- Wrote and graded questions for exams and homework assignments
- Earned second highest overall student evaluation of nine teaching assistants

Publications

Coufal M, Maxwell MM, Kearney S, Young A, Housman DE, Kazantsev A (2005). Mutant Huntingtin Cell-Based Clearance Assay. Poster presentation at MIT Cancer Center Retreat.

Coufal M, Maxwell MM, Kearney S, Young A, Housman DE, Kazantsev A (2003). Mutant Huntingtin Cell-Based Clearance Assay. Poster presentation at Gordon Conference on CAG Triplet Repeat Disorders.

Wong AK, **Alfert M**, Castrillon D, Shen Q, Holash J, Yancopoulus G, Chin L. (2001). Excessive tumor-eleborated VEGF and its neutralization define a lethal paraneoplastic syndrome. PNAS 98 (13):7481-6.

Works in Progress

Identification of Small Molecules Selectively Targeting Mutant Huntingtin

Discovery of Novel Small Molecule, Targeting Selective Clearance of Mutant Huntingtin Fragments in Cell