Discovery of a Novel Small-Molecule Targeting Selective Clearance of Mutant Huntingtin Fragments

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CAG-triplet repeat extension, translated into polyglutamines within the coding frame of otherwise unrelated gene products, causes 9 incurable neurodegenerative disorders, including Huntington’s disease. Although an expansion in the CAG repeat length is the autosomal dominant mutation that causes the fully penetrant neurological phenotypes, the repeat length is inversely correlated with the age of onset. The precise molecular mechanism(s) of neurodegeneration remains elusive, but compelling evidence implicates the protein or its proteolytic fragments as the cause for the gain of novel pathological function(s). The authors sought to identify small molecules that target the selective clearance of polypeptides containing pathological polyglutamine extension. In a high-throughput chemical screen, they identified compounds that facilitate the clearance of a small huntingtin fragment with extended polyglutamines fused to green fluorescent protein reporter. Identified hits were validated in dose-response and toxicity tests. Compounds have been further tested in an assay for clearance of a larger huntingtin fragment, containing either pathological or normal polyglutamine repeats. In this assay, the authors identified compounds selectively targeting the clearance of mutant but not normal huntingtin fragments. These compounds were subjected to a functional assay, which yielded a lead compound that rescues cells from induced mutant polyglutamine toxicity. (Journal of Biomolecular Screening 2007:351-360)

Key words: Huntington’s disease, high-throughput screening, huntingtin, protein clearance, toxicity rescue

INTRODUCTION

THE PRECISE MECHANISM(S) OF NEURODEGENERATION in Huntington’s disease (HD), caused by CAG triplet-repeat extension translated into polyglutamines, remains elusive.1 The native function(s) of the HD protein, huntingtin (Htt), and its role in pathogenesis are not yet clear.2 In mice, homozygous deletion of the HD gene results in embryonic lethality, whereas the hemizygous mice are viable.3,5 Some data suggest a neuroprotective role for wild-type Htt and a loss of native function caused by mutant Htt.6,8 However, Htt fragments of various lengths, when expressed as transgenes, are apparently sufficient to cause a progressive neuropathological phenotype. Mutant Htt fragments, which are proteolytic products of full-length protein, are implicated in a gain of novel pathological function(s).9 This conclusion is based on a large body of experimental evidence generated in cell and animal disease models.

Htt is ubiquitously expressed,10,11 but the striatum and cortex are the brain regions primarily affected in HD.12,13 The presence of insoluble inclusions, formed by aggregated mutant polypeptides with extended polyglutamines, has been detected in affected disease neurons in patients and in transgenic mice.14 The role of polyglutamine aggregates in the pathogenesis of HD remains unclear. In cell-based models, mutant Htt fragments form polyglutamine aggregates in a concentration- and time-dependent manner. Mutant polyglutamine polypeptides, which exist in both soluble and aggregated forms in the cell, pose a difficult model to study. In cells, soluble polyglutamines undergo an aggregation process consisting of 2 steps: slow nucleation and rapid polymerization.15 This process sets up a new equilibrium between the polypeptides in aqueous and solid phases. Furthermore, mutant polypeptides are synthesized de novo, and soluble and aggregated polyglutamines are subjected to degradation by cells, albeit at different rates.

Experiments with HD94 mice, inducibly expressing exon 1 Htt fragments, suggest that disease progression can be stopped and even reversed when expression of the mutant polypeptide...
is stopped.\textsuperscript{16-19} The amelioration of the neurological phenotype is associated with clearance of pathological-length polyglutamines, both soluble and aggregated, in affected disease neurons. This experimental clearance model implies that the inability of the degradation pathway(s) to sufficiently reduce levels of mutant polypeptides plays a role in disease pathology.

On the basis of published experimental data, we reasoned that small molecules that selectively target the extended polyglutamine domains within polypeptide sequences for degradation would likely provide therapeutic benefits, irrespective of the mechanism(s) of neurodegeneration mediated by mutant Htt polypeptides. Here we report a discovery of a novel compound, which facilitates the selective clearance of polypeptides containing pathological polyglutamine extensions and rescues polyglutamine-mediated toxicity in cells.

**MATERIALS AND METHODS**

**Cell culture**

HD103Q–enhanced green fluorescent protein (EGFP) PC12 cells,\textsuperscript{20} which inducibly express the Htt103Q-EGFP fusion protein, were grown at 37 °C with 5% CO\textsubscript{2} in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum, 2 mM L-glutamine, 200 µg/mL G418, and 100 µg/mL Zeocin.

Δ-α97Q and Δ-α23Q PC12 cells, which inducibly express both the β-galactosidase Δ fragment and the α fragment fused to either 97 or 23 polyglutamines, were generated using the Ecdysone-Inducible Mammalian Expression System (Invitrogen, Carlsbad, CA). Cells were grown at 37 °C with 5% CO\textsubscript{2} in DMEM supplemented with 15% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin. The pVgRXR, pIND/β-galactosidase Δ-subunit, and pIND/Hygro α 97Q or α 23Q constructs were maintained with 200 µg/mL Zeocin, 250 µg/mL G418, and 100 µg/mL Hygromycin, respectively. Expression of Δ-α97Q or Δ-α23Q in these cell lines was induced using either of the available ecdysone analogs, Ponasterone A (AG Scientific, San Diego, CA) or the more potent Muristerone A (Invitrogen).

Htt\textsuperscript{Q103} PC12 cells,\textsuperscript{21} in which induction of Htt Q103 results in cell death, were grown at 37 °C with 10% CO\textsubscript{2} in DMEM supplemented with 25 mM HEPES, 5% calf serum, 5% horse serum, 2 mM L-glutamine, and penicillin-streptomycin. The plasmid was maintained with 500 µg/mL G418.

**Western blots**

For preparation of protein extracts, HD103Q-EGFP PC12 cells were cultured in 24-well plates. Compounds were added, and expression of the HD103Q-EGFP fusion protein was induced by treatment with 1.25 µM Muristerone A (Invitrogen) at the time of plating. Cells were grown at 37 °C for 72 h. Proteins were extracted from cells with lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% [v/v] NP-40, 0.5% [w/v] Na-deoxycholate, 0.1% [w/v] sodium dodecyl sulfate [SDS], 5 mM EDTA) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1× Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) at 4 °C for 15 min. Protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Protein samples (20 µg each) were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immobilized on polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Billerica, MA). Blots were blocked in phosphate-buffered saline with Tween-20 (PBST) with 5% milk and incubated with primary antibodies against actin (1:1000, Sigma, St. Louis, MO) or polyglutamine (1:5000, Chemicon, Temecula, CA). Horseradish peroxidase (HRP)–conjugated secondary antibodies were obtained from Sigma. Proteins were visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Wellesley, MA), and blots were exposed to X-OMAT LS film (Kodak).

**Compound collection**

The in-house collection at the MassGeneral Institute for Neurodegenerative Disease consists of combinatorial compound libraries purchased from Chembridge Corp. (San Diego, CA; 30,000 compounds comprising the Diverse\textsuperscript{TM} and CNS\textsuperscript{TM} Sets) and from Maybridge Corp. (Cornwell, UK); 5000 diverse heterocyclic chemicals), together with 2000 natural products and a focused library (200) of aspartyl protease inhibitors, both purchased from TimTec Corp. (Newark, DE). Compounds were obtained from vendors in bar-coded 96-well microplates, each of which contains 80 unique compounds arrayed in 10 columns; the extreme left and right columns of each plate contain solvent only (DMSO), leaving the corresponding wells on assay plates available for assay controls.

**Assay design and high-throughput screen**

HD103Q-EGFP PC12 cells were used to develop a high-throughput screening (HTS) assay for compounds that promote clearance of extended polyglutamines. For assay development, cells were treated with various concentrations of the inducer Muristerone A, and EGFP fluorescence was analyzed at several time points (see Results). Z’ values were then calculated for each concentration of inducer at each time point using the following equation: $Z’ = 1 - (3\sigma_C + 3\sigma_C)/(\mu_C - \mu_C)$, where $\mu_C$ and $\mu_C$ are the mean maximum and minimum values of fluorescent signal, respectively, and $\sigma_C$ and $\sigma_C$ are the standard deviations of those means. For this assay, maximum signal was defined as fluorescence readings from cells induced to express 103Q-EGFP, and minimum signal was that obtained from unin­duced cells. By varying inducer concentration and incubation...
time, we were able to identify conditions that resulted both in $Z'$ values acceptable for screening and in an assay window that would permit us to reliably measure compound-dependent clearance of the HD103Q-EGFP fusion protein (see Results).

For HTS, HD103Q-EGFP PC12 cells were plated in cell culture-treated 96-well black microplates (PerkinElmer) at 2 to $4 \times 10^5$ cells/well using an automated cell dispenser (Multi-Drop, TiterTek, Huntsville, AL). The following day, HD103Q-EGFP protein expression was induced with 1.25 µM Muristerone A, and compounds were added to assay plates using a robotic liquid handler platform with a 96-well dispensing head (Evolution P3, PerkinElmer). On each assay plate, wells in the extreme left column received solvent only (without inducer) to control for background fluorescence, and wells in the extreme right column received solvent plus Muristerone A to provide the maximum signal obtainable. The middle 10 columns received compounds and Muristerone A simultaneously. For the primary screen, compounds were screened in triplicate at a single dose of ~5 µM. Following addition of compounds, assay plates were incubated for 72 h. Compounds were removed using an automated plate washer (BioTek), and cells were lysed in 150 µL lysis buffer prior to assay readout. Assay results were collected by reading sample fluorescence, using a Victor³-V multilabel plate reader (PerkinElmer).

**Compound dose-response tests**

HTS hit compounds were assayed for dose response in HD103Q-EGFP PC12 cells. Using the conditions established for the primary screening assay, hit compounds were restested over a range of compound concentrations from 0.1 to 35 µM to generate dose-response curves. Most hits were biologically active at concentrations ranging from 2.5 to 10 µM and were nonspecifically cytotoxic at higher concentrations.

**Compound cytotoxicity tests**

Hit compounds were assayed for cytotoxic effects in HD103Q-EGFP PC12 cells. These tests were performed using assay conditions similar to those established for the HTS, except that toxicity tests were performed using uninduced cells because clearance activity might influence readouts in this assay. The WST-1 reagent (Roche), a tetrazolium salt-based assay for metabolically active cells, was used to assess cell viability following compound treatment. Cytotoxic effects were identified by a dose-dependent decrease in cell viability, and compounds were tested over a range of compound concentrations from 0.1 to 35 µM to permit generation of $TC_{50}$ curves for all HTS hits. In addition, these studies were repeated using different batches of hit compounds than those used in the primary screen to ensure reproducibility of effects and to control for compound purity.

**β-Galactosidase activity assay**

For the β-gal assay, Δ-α 97Q or Δ-α 23Q cells were seeded at $5 \times 10^4$ cells/well in 96-well plates and induced with either Ponasterone A at 3 µM or Muristerone A at 1.25 µM, plus compound or solvent alone (DMSO), and then grown at 37 °C for 24 h. (These concentrations of the different inducers yielded equivalent results in this assay.) Cells were then rinsed with phosphate-buffered saline (PBS) and lysed by addition of 10 µL of modified radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris HCl [pH 7.4], 1 mM EDTA, 1% NP-40, 1% w/v Na-deoxycholate). Then, 67 µL of a master mix (5 µL 10× Cleavage Buffer, 0.135 µL 14.3 M β-mercaptoethanol, 44.865 µL dH₂O, 17 µL 4 mg/mL ortho-nitrophenyl-β-D-galactoside [ONPG]) from Invitrogen’s β-galactosidase assay kit was added to each well and incubated at 37 °C for 30 to 60 min. Then, 125 µL of stop buffer (1 M Na₂CO₃) was added to stabilize the colorimetric change of ONPG cleaved by β-galactosidase, which was then read at 405 nm on a plate reader.

The data from the experimental wells were normalized by subtracting the amount of β-galactosidase activity in uninduced wells. To report the percentage of β-galactosidase activity, 100% β-galactosidase activity was defined as the activity of induced cells that were not treated with compound, whereas 0% activity was defined as the activity of uninduced cells. Subsequently, all experimental data are reported relative to these 2 controls.

**Viability assay in HttQ103 PC12 cells**

Compounds were assayed for their ability to rescue polyglutamine-mediated cell death using HttQ103 PC12 cells.²¹ HttQ103 PC12 cells were seeded in 96-well plates at $2 \times 10^4$ cells/well and then grown at 37 °C for 72 h. Compounds were added and HttQ103 expression was induced at the time of plating. Cell viability was assessed using the MTS assay, a tetrazolium salt-based assay for metabolically active cells, which was performed using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer’s instructions.

**RESULTS**

**Assay design and HTS**

Experiments with the HD94-inducible mouse model¹⁶ suggest that the pathways degrading polypeptides containing extended polyglutamines are intact but inefficient in HD. We obtained similar results in PC12 cells expressing extended polyglutamines in an inducible fashion. In these cells, the N-terminal 17 aa of the Htt protein and extended polyglutamine repeats is expressed as a fusion with EGFP (HD103Q-EGFP).²⁰
We assessed the degradation of extended polyglutamines in experiments designed to mimic a pulse-chase assay. Cells were induced to express HD103Q-EGFP fusion proteins for 24 h, and then expression was stopped by inducer withdrawal. We detected rapid clearance of the HD103Q-EGFP fusion protein within 24 h of inducer withdrawal (Fig. 1). Within an additional 24-h period, cells cleared mutant polyglutamines to basal (uninduced) levels. Clearance of HD103Q-EGFP fusion proteins was assessed by immunoblotting with an antibody against extended polyglutamines (Fig. 1A) and was confirmed by probing with green fluorescent protein (GFP)-specific antibodies (data not shown). Degradation of HD103Q-EGFP fusion proteins correlated with the decline of green fluorescent signal (Fig. 1B). Hence, we determined that, in the absence of newly synthesized HD103Q-EGFP fusion proteins, cells cleared extended polyglutamines to the background level within 48 h. However, most HD103Q-EGFP proteins were cleared within the first 24 h after inducer withdrawal, followed by rapid decline of corresponding green fluorescent signal. These observations were confirmed by epifluorescent analyses of live cells (Fig. 1C-H).

To develop the assay for HTS, we tested the effects of different incubation periods and inducer concentrations on HD103Q-EGFP fluorescence levels and calculated the Z’ value of the assay for each condition tested (Fig. 2A and data not shown). Figure 2A shows that, when measured 72 h following addition of Muristerone A, the Z’ values for this assay are 0.23, 0.45, 0.52, and 0.5 at inducer concentrations of 0.5, 1.25, 2.5, and 5 µM, respectively. These results indicated that our assay was sufficiently robust using inducer concentrations ≥ 1.25 µM because a Z’ value ≥ 0.4 is typically considered acceptable for HTS.

To calibrate the screening assay further, we tested the effects of the transglutaminase inhibitor cystamine on assay readout (Fig. 2B). Because cystamine is a potent inhibitor of HD103Q-EGFP protein levels in these cells and thus mimics the effects of a hit compound that promotes polyglutamine clearance, we used it as a reference compound to optimize assay parameters for HTS. Cystamine caused a reduction in HD103Q-EGFP protein levels and a dose-dependent decrease in total EGFP fluorescence (Fig. 2B). These effects were most pronounced when HD103Q-EGFP expression was induced with 1.25 µM Muristerone A; using this inducer concentration, treatment with 25 and 50 µM cystamine decreased HD103Q-EGFP fluorescence by 35% and 65%, respectively. At higher concentrations of inducer, cystamine had less pronounced effects on assay readout.

The above results show that cells can rapidly clear polypeptides containing extended polyglutamines and that our assay readout is sufficiently robust for HTS. A critical consideration for determining parameters for screening was that the rapid degradation of HD103Q-EGFP fusion proteins observed upon removal of inducer might obscure the effects of compounds on polyglutamine clearance. Therefore, we wished to optimize the screening assay conditions such that compound addition occurs when HD103Q-EGFP synthesis and degradation occur at the same rate. We assessed the effects of incubating cells for 24, 48, or 72 h following induction using different concentrations of the inducing agent, Muristerone A (Fig. 2A). We then repeated the time course experiment using HTS assay conditions, that is, by plating cells in triplicate 96-well assay plates and inducing with 1.25 µM Muristerone A (Fig. 2C). We determined that HD103Q-EGFP expression levels increased for 48 h and then leveled off between 48 and 72 h after induction with 1.25 µM Muristerone A (Fig. 2A,C). These data were consistent with the 24-h half-life of Muristerone A activity. The plateau in HD103Q-EGFP fluorescence observed between 48 and 72 h after induction presumably reflects an equilibrium between de novo synthesis and degradation (clearance) of HD103Q-EGFP polypeptides, and it defines an appropriate window for measuring compound-dependent clearance of polyglutamines.

To maximize our chances of identifying compounds with modest activities, we opted to perform the screen using 1.25 µM Muristerone A, the lowest concentration of inducer that yielded a Z’ value for the assay. In addition, we sought to minimize the amount of inducer used in the screening assay to avoid a high rate of polyglutamine aggregation, a concentration-dependent process. This consideration is important because when proteins with extended polyglutamines aggregate, their rate of degradation is altered, and the effects of compounds on clearance are obscured. Once the aggregation process begins, essentially all HD103Q-EGFP polypeptides in the cell are rapidly recruited to a few large inclusions. These polyglutamine aggregates are highly resistant to degradation in our PC12 cell model. In cells treated for 72 h with 1.25 µM Muristerone A, aggregates formed only in 10% of cells, whereas more than 50% of cells contain aggregates at higher concentrations of Muristerone A (data not shown). We therefore selected the following parameters for our screen: the reporter protein was induced with 1.25 µM Muristerone A, compounds were added simultaneously, and cells were incubated for 72 h prior to measuring the effects of compounds on HD103Q-EGFP protein clearance.

The HD103Q-EGFP PC12 cell-based assay was screened against 37,000 synthetic compounds in our in-house screening collection (see Materials and Methods). The hit selection threshold was set to identify compounds that decrease EGFP fluorescence by at least 35% (> 3 standard deviations) as compared to induced but untreated controls (Fig. 2D). The cumulative results of screening the entire compound library are shown in Figure 2E. The primary hit rate was determined to be 0.38%. Screening hits were cherry-picked and subjected to preliminary dose-response and cytotoxicity tests at 5-, 10-, and 20-µM concentrations to eliminate false positives. In total, 114 hit compounds were selected and reacquired from the vendors for follow-up studies.
FIG. 1. Clearance of extended polyglutamines in HD103Q–enhanced green fluorescent protein (EGFP) PC12 cells. (A, B) HD103Q-EGFP PC12 cells were induced with 1.25 µM Muristerone A at 0 h and incubated for 72 h. In samples indicated with “w,” inducer was removed 24 h after induction. (A) “Pulse-chase” experiment to monitor degradation of extended polyglutamines in the HD103Q-EGFP PC12 cell line. Cell extracts were collected at 0, 24, 48, and 72 h after induction, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by Western blotting with antibodies specific to extended polyglutamines. Experimental samples were analyzed in duplicate, and blots were probed with actin to control for sample loading. (B) Degradation of HD103Q-EGFP fusion proteins was analyzed by fluorescent readout. Fluorescence was measured at 0, 24, 48, and 72 h after induction. n = 8; error bars indicate standard deviation. (C-H) HD103Q-EGFP cells were induced with 1.25 µM Muristerone A at 0 h and incubated for 72 h. In G and H, inducer was removed 48 h after induction (“w”). (C, E, G) Epifluorescent and (D, F, H) phase contrast images at (C-D) 0 h, (E-F) 72 h, or (G-H) 72 h “w”.
FIG. 2. Assay development and high-throughput screen (HTS). (A) The graph shows inducer concentration-dependent changes in HD103Q–enhanced green fluorescent protein (EGFP) fluorescence after incubation for 24 h (white bars), 48 h (gray bars), or 72 h (black bars). Assay $Z'$ values were calculated for each inducer concentration at the 72-h time point (see Materials and Methods). $n = 8$; error bars indicate standard deviation. (B) The effects of a reference compound, cystamine, on fluorescent assay readouts are shown. HD103Q-EGFP expression was induced with 1.25, 1.75, or 2 µM Muristerone A and incubated for 72 h with solvent (white bars) or cystamine (black bars) at the concentrations indicated. For each concentration of inducer used, reduction in fluorescent signal by 50% is indicated by a solid line. $n = 3$. (C) Time course of fluorescent response in HD103Q-EGFP PC12 cells cultured using HTS assay conditions (see Materials and Methods). Cells were plated in triplicate assay plates and treated with compound solvent (DMSO). HD103Q-EGFP expression was induced (where appropriate) by treating cells with 1.25 µM Muristerone A, and fluorescent readouts were measured at 24, 48, and 72 h after induction. Fluorescent signal in uninduced cells is set at 0 and that of induced cells at the 72-h time point at 100%. A 15% decrease from maximal signal was determined to be 3 standard deviations. The threshold for hit selection was set at 65% of maximal control signal and was used to identify primary hits. $n = 6$; error bars indicate standard deviation. (D) Compound effects on relative fluorescence levels from 1 set of triplicate 96-well assay plates are shown. Induced vehicle-treated control is set at 100% and uninduced vehicle-treated cells at 0%. Relative fluorescence values were averaged across triplicates, and means were used to generate hit list. A sample hit compound is circled. (E) Summary of HTS results.
Further testing and prioritization of HTS hits

Hits were further tested in more comprehensive toxicity and dose-response tests (see Materials and Methods). Toxicity and dose-response assays were performed in parallel, using assay conditions established for the primary screen, to facilitate comparison of TC₅₀ and EC₅₀ values (data not shown). In addition, because negative cell-based screens (in which hit compounds are expected to yield a reduced signal) are prone to select compounds with nonspecific cytotoxicity, we used dose-response and toxicity tests to filter out these types of potential artifacts. Compounds demonstrating cytotoxicity greater than 2 standard deviations from controls in the concentration range of 0.1 to 10 µM were eliminated from further analyses. In addition, we visually assessed live cells in the presence of compounds at 1, 5, and 10 µM and filtered out compounds with apparent effects on cell morphology. From these collective results, we prioritized 31 compounds from the 114 hits (Fig. 2E). We advanced this first priority group of 31 validated HTS hits for further testing in selected secondary assays. Below, we present data from 1 compound (A31) that performed favorably in all assays tested, along with data from a related compound (A28) for comparison.

Figure 3A,C shows the dose-response and toxicity curves for compounds A28 and A31, 2 close structural analogs (Fig. 3B) that were both identified as hits in the primary screen. The calculated EC₅₀ values for compounds A28 and A31 were 7.5 µM and 5 µM, respectively. As a next step, the effects of increasing doses of compound A31 on the clearance of HD103Q-EGFP fusion protein were analyzed both by epifluorescence (Fig. 3D-G) and Western blotting (Fig. 3H). Increasing dosage of compound A31 correlates with the decrease in levels of the HD103Q-EGFP fusion protein. A similar decrease in HD103Q-EGFP levels is observed following treatment with the related compound A28 (Fig. 3H) but not by an inactive compound (Fig. 3I). These data indicate that compounds belonging to the structural scaffold represented by A28 and A31 can promote a decrease in protein levels of small Htt fragments containing extended polyglutamines.

Secondary assay validation

To confirm the effect of compounds A31 and A28 on clearance of mutant Htt, we used a novel clearance assay based on β-galactosidase α-complementation. For this model, we generated an in-frame fusion of the small β-galactosidase α-subunit (α) fused at the N-terminus to a 547-aa fragment of Htt containing either mutant (97Q) or wild-type (23Q) polyglutamine tracts. The α-subunit was fused to the N-terminal region of Htt because this would allow for close monitoring of the integrity of the polyglutamine domain, which resides in the N-terminal region of Htt. The cells also express the β-galactosidase delta-subunit (Δ), which lacks the α-domain and is catalytically inactive. Both α-97Q and α-23Q fusions and the β-galactosidase α-subunit were stably integrated and expressed in PC12 cells under the control of the ec dysone-inducible promoter (see Materials and Methods). Upon intracellular α-complementation, cellular extracts gain β-galactosidase catalytic activity, which provides the basis for a highly sensitive and quantitative enzymatic assay. Alone, the small β-galactosidase α-subunit, lacking secondary and tertiary structure, is unstable in live cells. Therefore, degradation of the Htt component in the α-97Q and α-23Q fusion proteins subsequently causes degradation of the β-galactosidase α-subunit, which results in a reduction of α-complementation and leads to a decrease of assay readout signal.

Using this Δ-α 97Q and Δ-α 23Q cell-based assay, we observed an effect by compound A31 on the clearance of mutant, but not wild-type, Htt fragments (Fig. 4A). The effect of compound A31 was observed at 12 and 24 h after induction of expression, albeit at the reasonably high concentrations of 5 to 10 µM. The time course experiments were limited to 24 h, due to some cytotoxic effects of β-galactosidase overexpression observed at 36 h after induction (data not shown). These results demonstrate that compound A31 can mediate selective degradation of a large fragment of Htt containing extended but not normal polyglutamine tracts. In contrast, at concentrations up to 10 µM, compound A28 failed to reduce the reporter β-galactosidase in either Δ-α 97Q or Δ-α 23Q cells (data not shown). These results suggest that, at the modest concentrations tested, compound A28 is not active in cells expressing polyglutamines in the context of long fragments of Htt.

To determine if the decrease in toxic mutant Htt caused by compound A31 has an effect on cell viability, we tested this compound in a functional assay for polyglutamine-mediated cell death. For this purpose, we used HttQ103 PC12 cells, in which cell death is caused by inducible expression of a mutant exon 1 Htt fragment. In this model, induction of HttQ103 results in a 50% reduction in viable cells within 48 h. As shown in Figure 4B, compound A31 can rescue polyglutamine-mediated cell death in this system, although only at high concentrations.

DISCUSSION

An extension in the polyglutamine tract is the common mutation in 9 autosomal dominant neurodegenerative diseases in man. The mechanism of neurodegeneration is still unknown. However, it is evident from experiments with various animal and cell-based models that polypeptides containing extended polyglutamines are the direct cause of disease. We proposed a new therapeutic approach to intervene with the disease processes by using small molecules to target extended polyglutamines for degradation. Such an approach is similar in principle to reducing polyglutamine levels by other methods, such as RNA interference, which can target disease-specific polymorphisms. The therapeutic effect of small molecules, however, could be universally applicable for all polyglutamine diseases.

In the course of this work, we developed a mutant polyglutamine clearance assay, performed an HTS, and further tested
FIG. 3. Hit confirmation. (A) Dose-response assay showing the effects of A28 (gray bars) and A31 (black bars) compounds on fluorescent readout in the primary screening assay. HD103Q–enhanced green fluorescent protein (EGFP) PC12 cells were induced with 1.25 µM Muristerone A and treated with compound for 72 h. EC₅₀ values for A28 and A31 were determined to be 7.5 µM and 5 µM, respectively. (B) Structures of hit compounds A28 and A31. (C) Cytotoxicity data for compounds A28 (dashed line) and A31 (solid line) in HD103Q-EGFP PC12 cells. Uninduced cells were treated with compound for 72 h and assayed for cell viability (see Materials and Methods). Compound A31 did not reduce viability by more than 2 standard deviations from untreated control at concentrations ranging from 0.1 to 10 µM. The y-axis indicates percent viability compared to untreated control cells (set at 100%). (D-G) Epifluorescent images of cells treated with (D) DMSO or (E) 2.5 µM, (F) 5 µM, or (G) 10 µM compound A31. (H-I) Western blot analyses of compound-dependent extended polyglutamine clearance. Extracts from induced cells treated with compounds (H) A28 or A31 or (I) inactive compound control at concentrations of 1, 5, and 10 µM were analyzed using antibodies specific to extended polyglutamines. Experiments were performed in duplicate. Arrows labeled poly-Q indicate HD103Q-EGFP polypeptide. β-Actin was used as loading control.
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FIG. 4. Hit validation in secondary assays. (A) Δ-α 97Q (black bars) and Δ-α 23Q (white bars) cells were induced with 3 µM Ponasterone A and treated with 2.5 µM, 5 µM, or 10 µM compound A31 for 12 or 24 h and then assayed for β-galactosidase α-complementation (see Materials and Methods). The y-axis indicates β-galactosidase activity relative to induced cells without compound (set at 100%). (B) Relative survival of Htt97Q cells induced and treated with compound A31 (gray bars) for 72 h, compared to uninduced cells (0 µM compound, white bar, 100% viability) and induced cells without compounds (black bar, maximum toxicity). The y-axis indicates percent viability, as measured by the MTS assay, relative to uninduced cells without compound (0 µM, white bar). n = 12; error bars indicate standard deviation.

and validated hits in relevant secondary assays. This study yielded the lead compound A31, which facilitates the clearance of polypeptides containing the N-terminal 17 aa of Htt and an extended polyglutamine tract. This compound also rescued mutant polyglutamine-mediated cell death in HttQ103 cells that express exon 1 of Htt. Furthermore, compound A31 promoted clearance of a large Htt fragment (the first 547 aa) containing extended polyglutamines in Δ-α 97Q cells, as evidenced by reduced activity of the β-galactosidase reporter following compound treatment. The effectiveness of compound A31 in these independently derived cell lines, expressing different-sized fragments of Htt, strongly argues for pursuing this compound as a potential treatment for HD.

Our screening collection contains 4 additional structures that are closely related to A31 and A28; none of these was detected as a hit in the primary screen. In addition, we retested these 4 compounds, along with 11 other analogs of A31, in the primary screening assay, and all were either inactive or less effective than A31 and A28 (data not shown). We believe that the fact that some compounds similar to A28 and A31 failed to promote clearance of HD103Q-EGFP demonstrates the selectivity of the primary screening assay. The activities of the other hit compounds identified in the primary screen, which are structurally diverse from A31 and A28, are currently under investigation.

In contrast to compound A31, the close structural analog A28 failed to promote clearance of a large (547 aa) fragment of Htt containing either extended or wild-type polyglutamine tracts (data not shown), but it was active only against polyglutamines in the context of Htt exon 1. One possible explanation for this difference in the activities of A31 and A28 is that the additional sequence of Htt expressed in the β-gal assay reduced the ability of A28 to target these polypeptides for clearance, perhaps due to differences in protein conformation between the long and short Htt fragments. Another possibility is that clearance in this assay might require higher concentrations of A28, which was less potent than A31 in the primary screening assay (Fig. 3A). In this event, the clearance-promoting effects of A28 in the β-gal assay might be obscured, due to the fact that A28 exhibits appreciable cytotoxic effects in both of these assays at concentrations > 5 µM (Fig. 3C and data not shown). In limited structure-activity relationship (SAR) studies performed to date, we have been unable to develop an analog that exhibits greater potency than A31, and these efforts are ongoing.

In addition to its ability to promote clearance of both long and short fragments of the huntingtin protein, compound A31 exhibits remarkable selectivity for mutant versus wild-type Htt. The A31 compound has been tested in cell models expressing polypeptides containing normal polyglutamine repeats of 23Q and elongated repeats of 97Q or 103Q. The data show that compound A31 has no effect on Htt with normal polyglutamine length. On the other hand, compound A31 reduces the protein levels of Htt with expanded polyglutamines and rescues toxicity caused by expanded polyglutamine. It is crucial to determine the lengths of polyglutamines targeted for clearance by this small molecule because quite a few cellular proteins also contain polyglutamine stretches. In light of this consideration, it was important to demonstrate the lack of cytotoxicity associated with the lead compound, indicating that this compound most likely does not target other proteins containing normal lengths of polyglutamine tracts.

Compound A31 is effective in promoting the clearance of mutant polyglutamines within both short and long Htt protein sequences. Some published data suggest a correlation between short Htt fragments and neurotoxicity. However, not only are larger Htt fragments able to be processed into smaller fragments by proteolytic cleavage, but they themselves also may cause some detrimental effects on neurons. Therefore, we speculate that developing clearance assays similar to the β-galactosidase assay reported here, which target polypeptides with proper tertiary structures, is a more fruitful approach for identifying compounds that promote protein degradation.

We plan to advance the newly identified lead compound A31 for potency and ADMET optimization, to perform subsequent pharmacokinetic studies, and to test its efficacy in a mouse model of HD. The R6/2 transgenic mouse, expressing ~140 polyglutamine repeats within exon 1 of Htt, appeals as the most proper model for efficacy trials.

The results of this study demonstrate the feasibility and validity of a novel therapeutic approach, designed to identify compounds that promote clearance of expanded polyglutamines. We
have developed and characterized an array of assays, targeting selective clearance of mutant huntingtin fragments, that can be applied in high-throughput chemical screens to identify therapeutic leads for HD. The same array could be used in genetic screens, using RNAi or cDNA overexpression, to elucidate the precise mechanism(s) of action of hit compounds and to identify the cellular modalities that affect the clearance pathway(s). In addition, this approach is broadly applicable as a drug discovery tool, in that the same principle can be adapted for assays targeting mutant conformations of other gene products that cause dominantly inherited human neurological disorders.

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