Project Title: Aggregation and Clearance of Huntingtin Inclusions: Huntington's Disease Assay

Grant Number: 1R03MH076348-01

Screening Center Name: MLSCN Center at Columbia University

Principal Investigator of Screening Center: James Rothman

Assay Submitter & Institution: Ai Yamamoto, Columbia University

Assay or Pathway Target: Clearance of inclusions

Probe PubChem Compound Identifier (CID): 23631972

Assay provider information

Specific Aim: Huntington's disease (HD) is one of a series of polyglutamine (polyQ) disorders and is caused by the expansion of polyglutamine- encoding CAG repeats in the first exon of the HD gene encoding the huntingtin protein (Htt).[1,2,3] Expression of the mutant huntingtin protein (mHtt) results in the onset of a gradual, progressive, neuropsychiatric, degenerative illness, which invariably leads to death.[4,5] Recently, an exciting theme has emerged: if mutant protein accumulation is eliminated, symptomatic progression not only halts but also leads to recovery from the disease.[4] Unfortunately, the pathway underlying clearance of these mutant proteins has not been identified yet. Both proteasome- and lysosome-mediated degradation have been implicated to play a role in the clearance of expanded polyQ proteins. Screening of a well-designed cell-based assay with a compound library that would allow us to further clarify which degradative pathway is important as well as to identify new means by which these pathways can be activated was required. Thus, a functional fluorescent cell-based assay was developed to visualize and analyze aggregation and clearance of mutant huntingtin protein. The screen uses a stable HeLa cell line that conditionally expresses the first 17 amino acids of Htt followed by a polyQ stretch of 103 residues fused to a monomeric Cyano Fluorescent Protein (CFP) on their C-termini. This cell line permits high-throughput confocal microscopy to examine the state of the expressed mutant protein Cells are incubated with compounds for 3 days (72 h) to allow differences in amount of aggregate to become apparent. High-throughput confocal microscopy is used to examine the state of the expressed mutant protein in live or fixed cells.

Significance and Rationale: The recurrent observation of accumulation and aggregation of mutant proteins in different neurodegenerative disorders prompts the hypothesis that there may be a shared pathogenic mechanism. Animal models of HD[6,7,8] and spinocerebellar ataxia type 1[9,10] revealed that cells have the ability to clear the polyQ aggregates, if the continuous production of the mutant transgene is halted.[4] This clearance is accompanied by reversal of the disease-like symptoms in the tested mice[4]. These findings suggest that that if the mutant protein can be cleared, the disease can be reversed.

The complete molecular mechanism that drives clearance is still unknown. Experiments have shown that loss of mutant huntingtin protein expression can lead to clearance of both the soluble and aggregated forms of the protein.[6] By defining the pathways responsible for the clearance of the mutant protein, we can accelerate the rate of turnover of mutant huntingtin and thereby mimic the effects demonstrated in the mouse model. Finding the modifiers of these pathways will allow us to identify targets for treatment of this disease. This may contribute to the treatment of other neurodegenerative disorders which have mutant protein aggregation at their core.[11]

Screening center information

Assay Implementation and Screening

PubChem Bioassay Name: Aggregation and Clearance of Mutant Huntingtin Protein

List of PubChem bioassay identifiers generated for this screening project (AIDs): 483, 486

Primary Assay Description as defined in PubChem:

The neurodegenerative disorder Huntington's disease is caused by a polyglutamine expansion mutation in the HD gene that leads to a complex neurological disorder involving deterioration of psychiatric, cognitive and motoric function. Under nonpathogenic conditions, the N-terminus of the huntingtin protein (Htt) contains a stretch of glutamines (Q) that ranges from 2 to 34 repeats, while numbers of greater than 37 repeats invariably lead to the pathogenic outcome. Suppressing mutant Htt expression in an inducible mouse model of Huntington's led to complete recovery of symptomatic mice[6], and this reversal was tightly dependent on the clearance of the mutant protein. Can the elimination of the accumulated proteins be a means to bring about recovery in HD? A fluorescent cell-based assay was developed to analyze aggregation and clearance of mutant huntingtin protein.[4] The basis of this assay is a stable cell line that expresses the N-terminus of the huntingtin protein in an inducible manner with 103 glutamine repeats fused to monomeric CFP at the C-terminus. The end point of this assay measures the presence/absence of fluorescent intracellular inclusions. This readout allows for the use of high-throughput confocal microscopy to examine the state of the expressed mutant protein in live or fixed cells[4], and therefore screening of small molecules libraries can be performed.

Aggregation and Clearance of Mutant Huntingtin Protein HCS/HTS was developed and run at the Columbia University Molecular Screening Center as part of the Molecular Library Screening Center Network (MLSCN). Grant number: 1R03MH076348-01; Aggregation and Clearance of Mutant Huntingtin Protein**,** Assay Provider: Drs. A. Yamamoto and JE Rothman, Department of Physiology and Cellular Biophysics at Columbia University.

Center Summary of the Primary Screen:

Protocol:

Material:

For the culture and the screen

- Established cell lines from Dr. Ai Yamamoto: exon1htt(103Q)mCFP (frozen stock in cell bank)
- Trypsin/EDTA (GibcoBRL Invitrogen, #25300-112)
- DMEM (GibcoBRL Invitrogen, #11960-077)
- Hygromycin B (BD Biosciences, 50 mg/mL)
- Genetycin (G418) (Invitrogen)
- Compound library
- Positive control: Doxycycline (Sigma, #D9891-1g) [100 ng/mL final concentration]
- Paraformaldehyde (EMS, #15710 10x10 mL)
- Packard Plates (Perkin Elmer, #6005182) containing cells ready to be processed

For the Detection:

- Phosphate-buffered Saline (PBS), tables, w/o Ca^{2+} and Mg^{2+} (MP Biochemicals, #2810305)
- Hoechst 33342 (Molecular Probe Invitrogen, #H3570, 10 mg/mL solution in water)

Working solution:

- Fixation Solution: 4% Paraformaldehyde in PBS
- PBS
- Hoechst 33342 ,: 50 µL per well x 100 x number of plates + an additional 10 %)

Procedure:

Cell Seeding

- Cells are cultured in DMEM supplemented with 50 µg/mL Hygromycin and 50 µg/mL G148
- Seed 2.0 x 10^3 cells per well in 96 well “ViewPlates” in 100 µL of DMEM (supplemented with 50 µg/mL Hygromycin and 50 µg/mL G148) (“assay plates”)
- Incubate overnight (15h) at 37 ºC, 5 % CO_2 and humidity before screening

Compound Addition
- Dispense 5.5 µl of a compound solution 20x times concentrated than the final data point dilution (and positive and negative controls per well of "assay plates")
- Incubate 3 days 37 °C, 5% CO_2 and 100 % humidity
- Proceed to fixation
Fixation/Staining

- Dispense 50 µl fixation solution
- Incubate for 20 min at RT
- Aspirate
- Dispense 50 µl of Hoechst solution (1:10,000 in PBS)
- Incubate 20 min RT
- Wash 2X
- Dispense 100 µl PBS
- Plates are ready to be scanned

Analysis

6 compounds were confirmed as hits from the original 10K library screening (primary **AID 483**; confirmatory **AID 486**). One of these hits (**SID 4264628**) was selected for analog follow-up and SAR studies. In this publication, we report 38 analogs of the original compound that show inhibitory activity when run against the original assay, plus other compounds from the literature that were reported as autophagy inhibitors.[12]

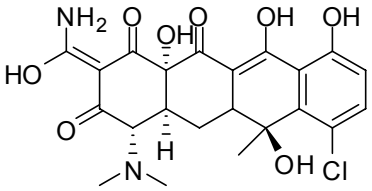
Image analysis of experimental results was done using the Granularity Analysis Module (GRN0), from INCell3000 software (GE healthcare) reporting the scaled measure of the fraction of cellular fluorescence present in the qualifying grains (Fgrains), and the nuclei/cell count (Npass).

All calculations were done using Assay Explorer 3.0 from MDL.

The analog compounds were assessed in dose-response experiments (triplicates, 6 data points log-based dilution starting at a concentration of 10 µM). For each compound, percentage inhibitions were plotted against compound concentration. A four-parameter equation describing a sigmoid dose-curve response was then fitted with adjustable baseline using Assay Explorer software. The reported IC_{50} values were generated from fitted curves either by determining the X-intercept of the inflexion point of the generated sigmoid curve (IC50_curve fit) or by solving for X-intercept at the 50% inhibition level of Y-intercept when a plateau was not reached (IC50_backcalc). In cases were the highest concentration tested (i.e. 10 µM) did not result in greater than 50% inhibition, the IC_{50} was determined manually as greater than 10 µM (IC50_manual).

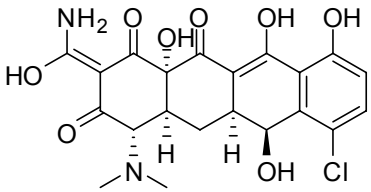
Identified Hits:

CID 6603543, SID 855713:



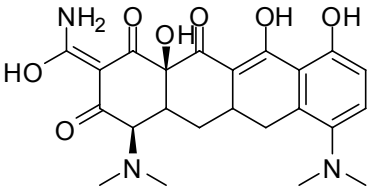
H-Cl

CID 6602612, SID 855598:



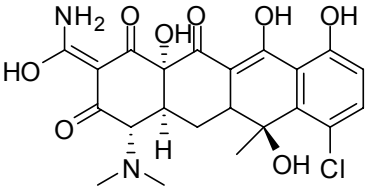
H-Cl

CID 6602603, SID 855650:

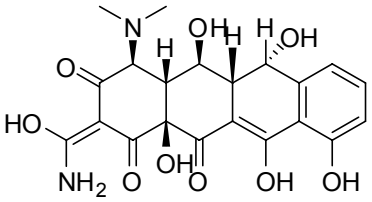


H-Cl

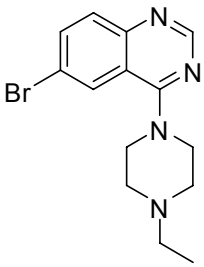
CID 5388997, SID 855950



CID 5282186, SID 855768:



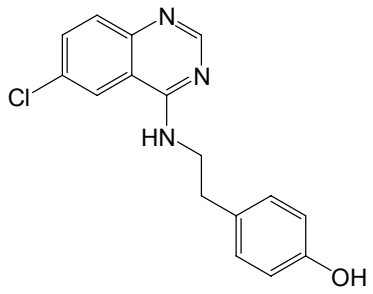
CID 720660, SID 4264628 → compound name: 6-bromo-4-(4-ethylpiperazin-1-yl)quinazoline:



<u>Probe Optimization</u>

Chemical name of probe compound: 4-(2-(6-chloroquinazolin-4-ylamino)ethyl)phenol

Draw probe chemical structure and show stereochemistry, if known:



Describe mode of action for biological activity of probe: Inhibition of aggregate formation

Has this compound been provided to the MLSMR: not yet, but will be ASAP

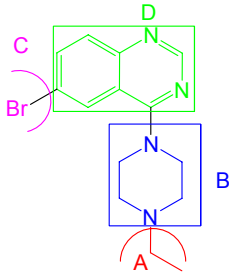
Description of secondary screens used to optimize probe structure:

It is necessary to distinguish between compounds that clear aggregates and compounds that inhibit aggregate formation. In the latter case, mHtt is still present in the cell, but in a dispersed form. Analogs displaying inhibitory activity were subjected to further analysis as follows. To investigate the source of the inhibition of the aggregate formation, we set up a series of counter screens to examine the integrity of the cytoskeleton and as well as that of the microtubules. It is in fact known, that an intact cytoskeleton is essential for inclusion body formation around the microtubule organizing center. For this purpose, we used phalloidin staining to visualize the distribution of F-actin in the cells, and an antibody against beta-tubulin to test for microtubule integrity. To rule out compounds that might compromise general protein sorting and modification, we followed the localization of p230 by immunostaining. p230 is a TGN (trans golgi network)-specific protein that is important for the biogenesis of distinct populations of non-clathrin-coated vesicles and, thus, plays a role in vesicular transport from the TGN. All of our active compounds showed no signs of disturbing these cellular pathways (data not shown).

Center comments on chemistry strategy leading to probe identification:

Five of the six hits were tetracycline compounds and served as positive controls rather than as viable hits. 6-Bromo-4-(4-ethylpiperazin-1-yl)quinazoline was the only viable hit.

Looking at the structure of the hit, we determined that we had four sites that could be modified (**Figure 1**). First, we could change the substituent on the piperazine ring (**Figure 1 Region A**) Second, we could remove the piperazine moiety and replace it with another kind of amine or alcohol (**Figure 1**, **Region B**) Third, we could change the halo substituent at the 6-position and replace it with either a different halogen or a hydrogen (**Figure 1, Region C**). Fourth, we could replace the quinazoline core with a 5*H*-pyrimido[5,4-*b*]indole, **I**, or a 7-methylthieno[3,2-*d*]pyrimidine, **II**, or a 5-methylthieno[2,3-*d*]pyrimidine, **III,** core (**Figure 7**).



CID 720660
SID 4264628

Figure 1

The original hit, **SID 4264628**, was determined to have an IC_{50} value in the low micromolar range (**Figure 1**). When this compound, **1a**, was synthesized by our group, an IC_{50} value of 2.21 μM was observed (**Figure 2**). Its chloro analog, **1b**, was slightly less active with an IC_{50} value of 3.63 μM (**Figure 2**). As the size of the substituent at the 6-position decreased, so did the activity of the respective analog, giving an IC_{50} value of 5.26 μM for the fluoro analog, **1c**, and an IC_{50} value of greater than 10 μM for the parent compound, **1d**. At this point we cannot make any statement about how the different halo substituents affect the activity of a given compound, since this trend is not universal for all of the compounds in our series. In a lot of cases only the chloro analogs appeared to be active (**Figures 2-4**). It is noteworthy that quinazoline itself is inactive in our assay.

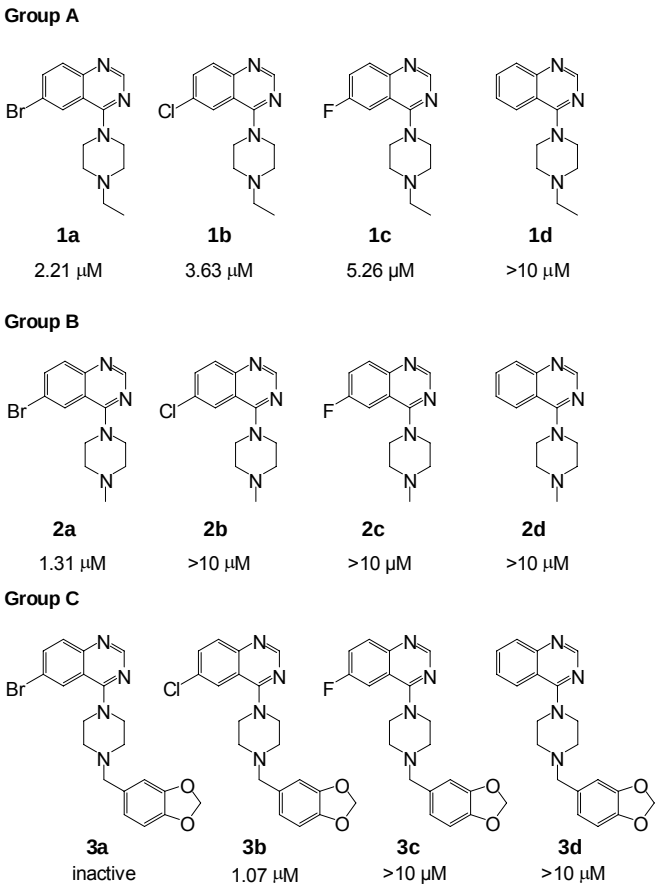


Figure 2: Series 1: Piperazine Analogs

When comparing analogs **1b** and **2b** we see a decrease in activity from 3.63 μM to greater than 10 μM with the loss of one methylene group (**Figure 2**). Only the activity of the bromo compound, **2a**, shows an increase in activity; compound **2a** has an IC_{50} value of 1.31 μM in contrast to compound **1a**, which has an IC_{50} value of 2.21 μM. Both substitution of the piperazine ring with a piperidine ring and replacement of the amino group with an alkoxy group resulted in complete loss of activity (**Figures 3** and **5**). Any substitutions of the quinazoline core with a 5*H*-pyrimido[5,4-*b*]indole, **I**, or a 7-methylthieno[3,2-*d*]pyrimidine, **II**, or a 5-methylthieno[2,3-*d*]pyrimidine, **III,** core resulted in loss of activity (**Figure 6**).

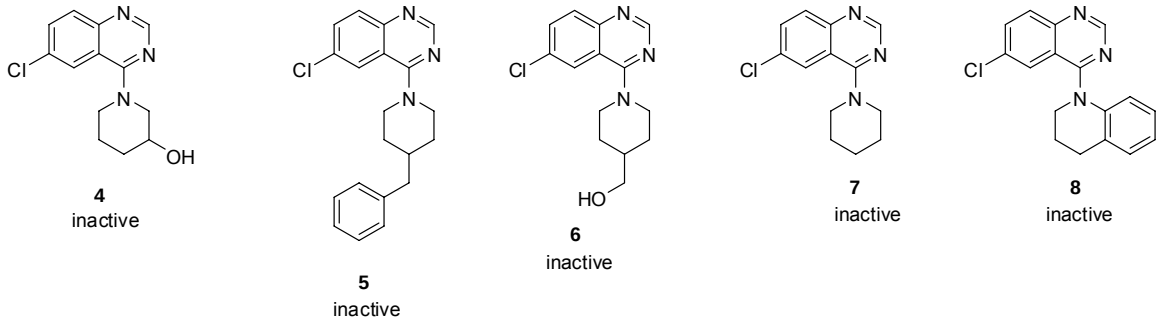


Figure 3: Series 2: Piperidinyl Analogs

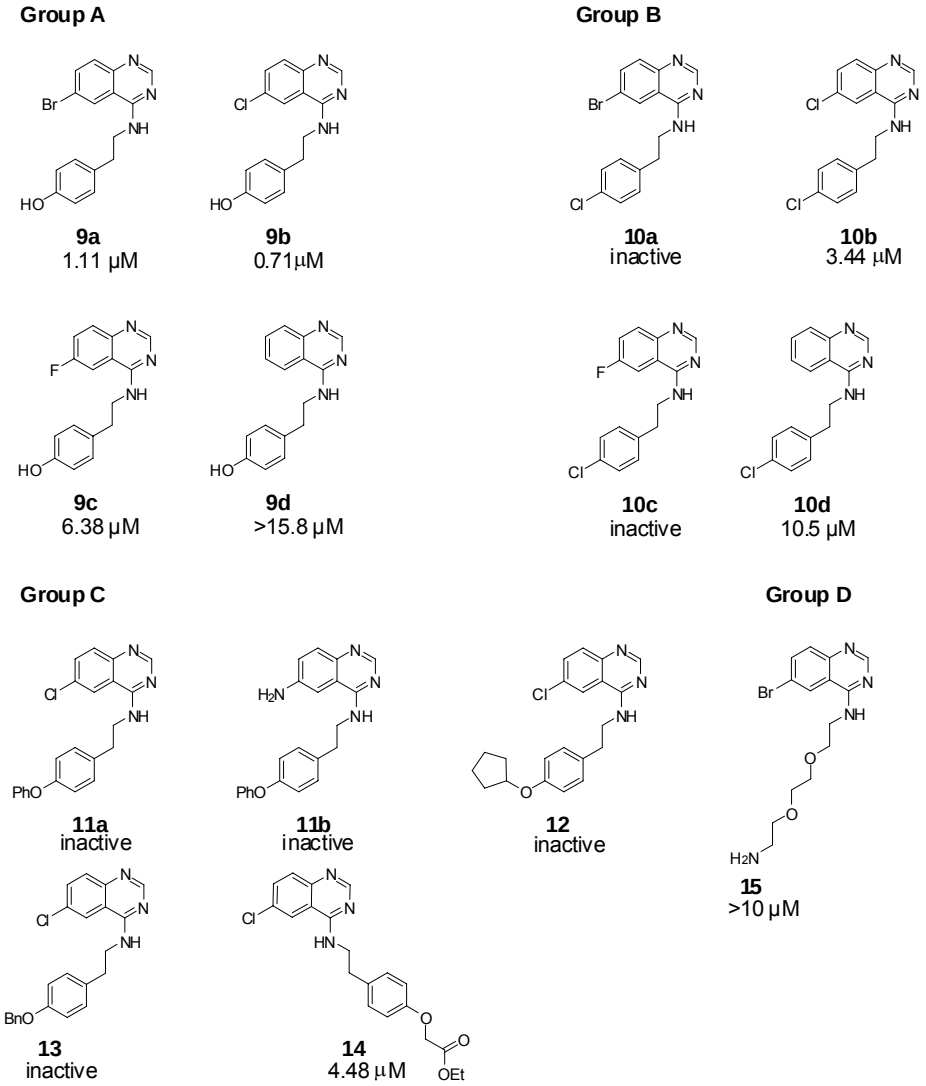
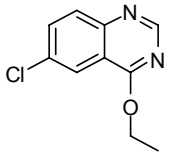
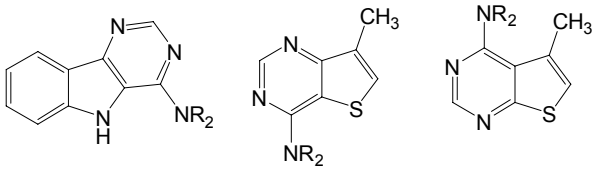


Figure 4: Series 3: Secondary Amine Analogs



16
inactive

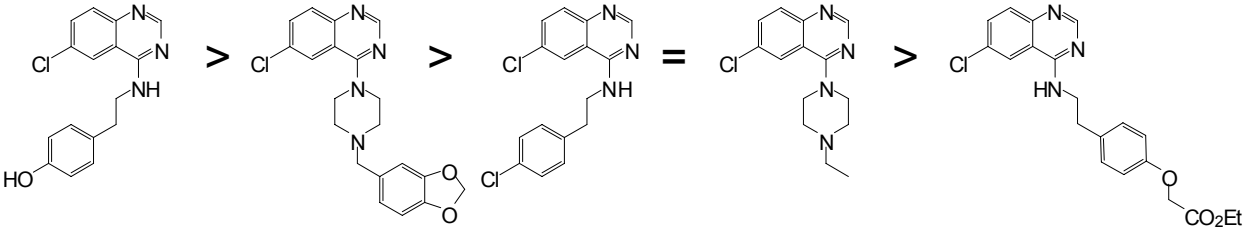
Figure 5: Alkoxy Analog



I **II** **III**

Figure 6: Analogs with Different Cores

Figure 7 displays the rank order of our active chloro analogs. Compounds **10b** and **1b** are approximately equal, as the difference between their IC_{50} values falls within the range of standard deviation. This rank order demonstrates how different modifications influence the activity of the given compound and gives us a better idea of what the binding pocket might look like. The IC_{50} curves of these compounds are shown below (**Diagrams 1-5**).



9b 0.71 μM > **3b** 1.07 μM > **10b** 3.44 μM = **1b** 3.63 μM > **14** 4.48 μM

Figure 7: Rank Order of our Active Chloro Analogs

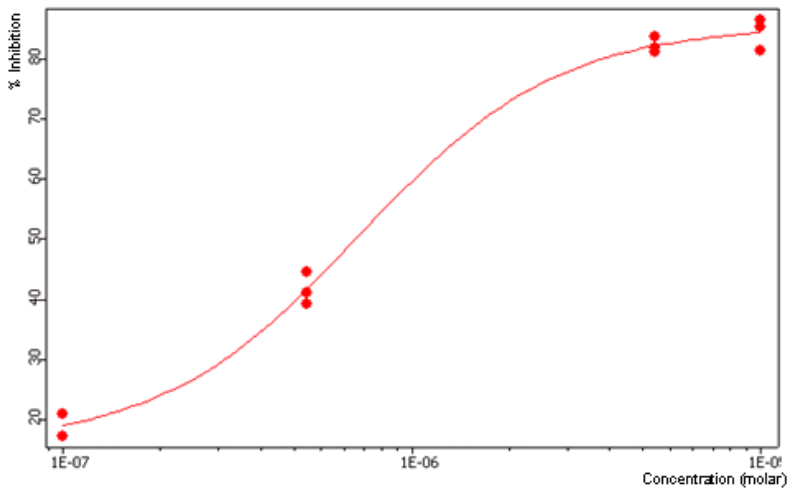


Diagram 1: IC_{50} Curve of Our Probe Compound, 9b

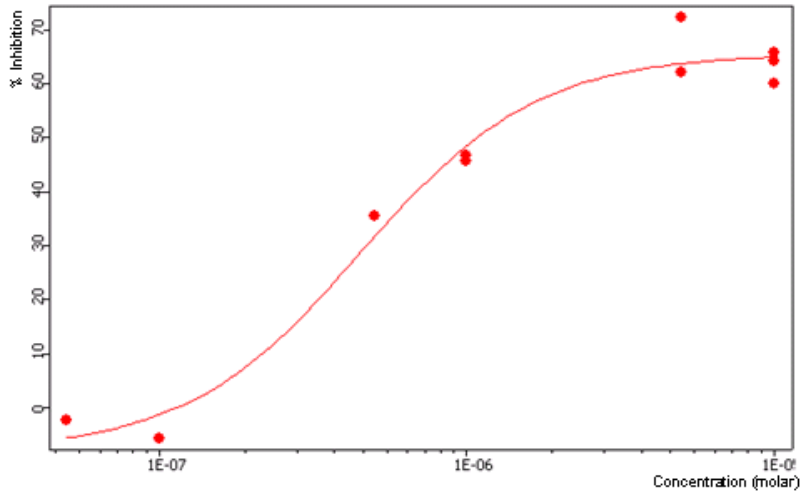


Diagram 2: IC_{50} Curve of 3b

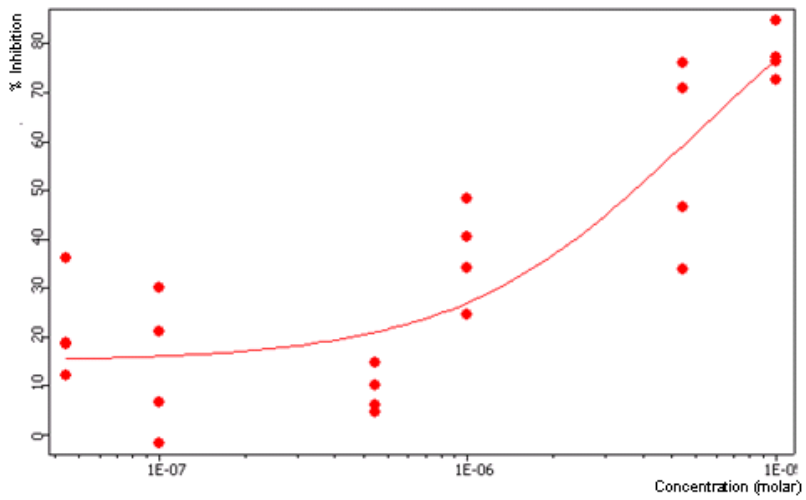


Diagram 3: IC_{50} Curve of 10b

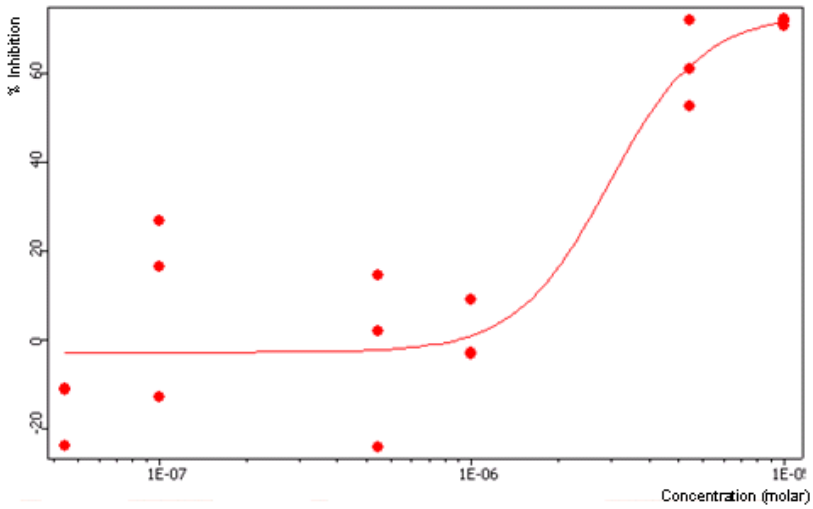


Diagram 4: IC_{50} Curve of 1b

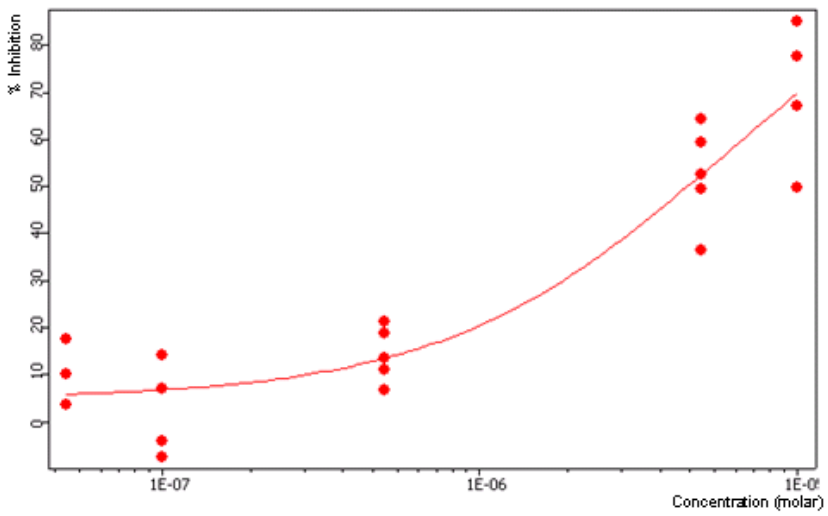


Diagram 5: IC_{50} Curve of 14

Sarkar *et al.* also found a bromo-substituted quinazoline to reduce aggregation and cell death in COS-7 cells caused by huntingtin exon1 with a repeat of 74 glutamines.[12] Since some of the structures were similar to ours, we decided to synthesize four of their compounds, namely compounds **17**, **18**, **19**, and **20**, to test in our cell-based assay (**Figure 8**). Although no EC_{50} values were reported in the published results for the four compounds, the authors indicate that the potency of their quinazoline analogs compared to the allyl compound, **18**, is less. The rank order of the potency for the four compounds in their system differed from that in ours and suggests different targets. For example, quinazoline **18** was reported to be one of their best compounds, yet it only showed minor activity in our assay (IC_{50} = 5.36 μM).

Our functional cell-based assay is based upon the clearance of the mutant protein in a stable cell line. This means that we do not observe the acute, polyQ-dependent cell death that can be monitored in other transiently transfected cell-based studies, such as the one used by Sarkar *et al.* Our assay allows for identification of hits for pathways other than recovery from cell death. Nonetheless, we acknowledge the fact that neuronal cell death is a critical aspect of HD and other polyQ diseases and should be considered when understanding findings in the context of the disease.

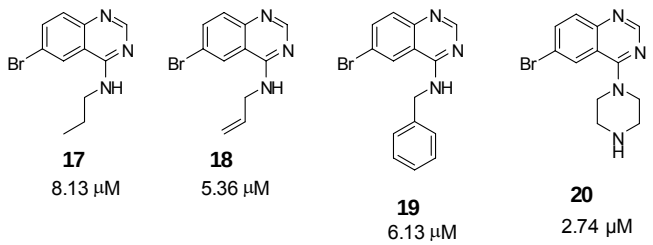


Figure 8: Enhancers of Autophagy reported by Sarkar *et al.*[12]

In addition to these quinazoline analogs we tested polyphenols[13,5], **21**-**24**, sulfobenzoic acid derivatives[1], **25**, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor[5], **26**, a nitric oxide donor[5], **27**, and todralazine hydrochloride[5] (**28**) (**Figure 9**). All of these compounds have been reported to have an effect in various neurodegenerative disease assays. In our assay the polyphenols were found to be either toxic or inactive. The bromo analog, **25** (X = Br), of the sulfobenzoic acid derivatives had been reported to interfere with polymerization of polyQ but not to inhibit nucleation both *in vivo* and *in vitro* when the protein is acutely expressed in PC12 cells.[1] Neither one of the sulfobenzoic acid derivatives was active in our assay. The EGFR tyrosine inhibitor, **26**, and the nitric oxide donor, **27**, were both inactive in our assay. Todralazine hydrochloride (**28**) was found to be toxic.

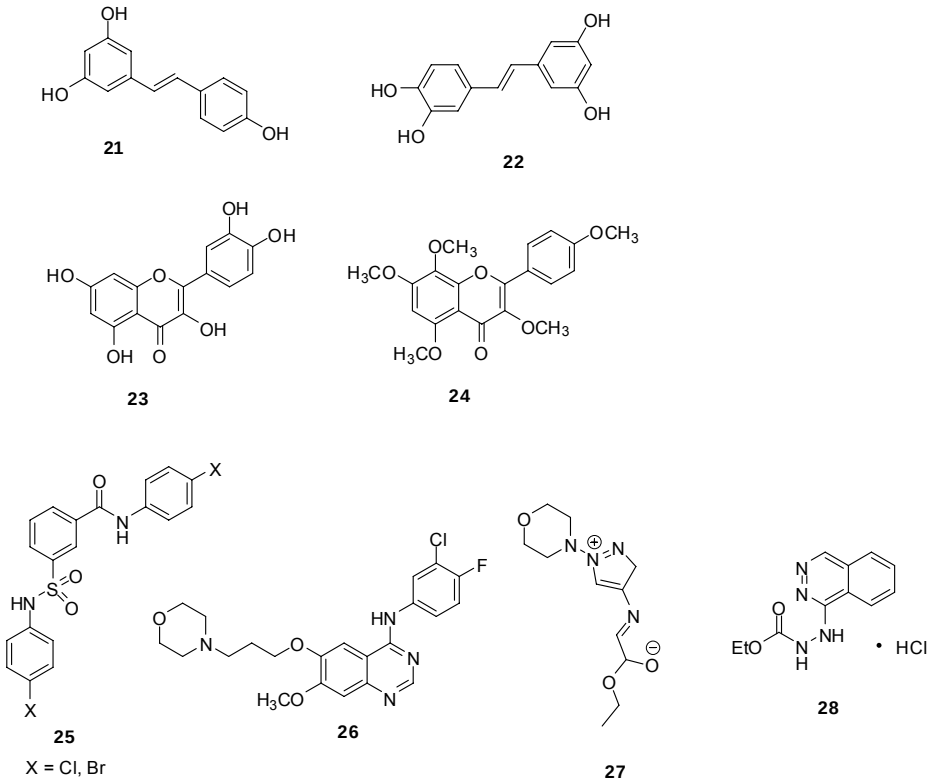
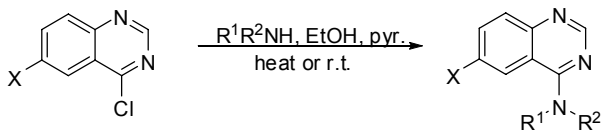


Figure 9: Literature-reported biologically active compounds in neurodegenerative disease assays

In summary, we prepared and identified four very potent inhibitors, **9b**, **3b**, **9a**, and **2a** (**Figures 2** and **4**). Quinazoline **9b** is our best compound with an IC_{50} value of 0.71 μM. Thus, this is our probe for this assay; it fulfills all of the requirements of a probe set forth by the NIH (**Figure 4**; **Diagram 1**).

Detailed synthetic pathway for making probe:



29 **30**

X = Br, Cl, F, H

Scheme 1

General procedure:

^{1}HNMR and ^{13}C NMR spectra were recorded on a Varian Mercury 300 T NMR spectrometer. Chemical shifts are reported in parts per million using residual proton or carbon signal ($(CD_3)_2SO$: δ_H 2.50, δ_C 39.52) as an internal reference. The apparent multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), and coupling constants (in Hz) are reported in that order in parentheses after the chemical shift. All melting points were determined on a Mel-Temp II Laboratory Devices apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on EMD Chemicals Inc. regular TLC plates. Developed TLC plates were visualized by a 254-nm UV lamp and/or iodine. Flash chromatography refers to the procedure developed by Still[14] employing 230-400 mesh Silicycle® silica gel. Mass spectra were performed on a Shimadzu LCMS-2010 A liquid chromatograph mass spectrometer. High-resolution mass spectrometry was done by Dr. Yasuhiro Itagaki at Columbia University.

4,6-Dichloroquinazoline (**29**, X = Cl) was stirred at room temperature in a 4:1 mixture of ethanol (4.0 mL) and pyridine (1.00mL). Tyramine (0.124 g, 0.904 mmol) was added to the white suspension which immediately upon addition of the amine turned dark bright orange and then gradually turned into a clear, bright orange solution. The solution was stirred overnight and was subsequently heated for 5h at 50 ºC, after which it was allowed to cool to room temperature. The product was purified via column chromatography (1:1 hexanes : ethyl acetate) to give a white solid, **9b**, (0.106 g, 0.371 mmol, 62 %): mp 178-180 °C; ^{1}H NMR (300 MHz,$(CD_3)_2SO$) δ 9.19 (s, 1H), 8.49 (s, 1H), 8.39 (s, 1H), 7.78 (d, 1H, *J* = 9.0 Hz), 7.69 (d, 1H, *J* = 8.7 Hz), 7.05 (d, 2H, *J* = 8.1 Hz), 6.68 (d, 2H, *J* = 8.4 Hz), 3.67 (t, 2H, *J* = 7.5 Hz), 2.83 (t, 2H, *J* = 7.5 Hz); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) δ 158.4 (1C), 155.5 (1C), 155.4 (1C), 147.6 (1C), 132.7 (1C), 129.6 (2C), 129.4 (2C), 129.3 (1C), 121.9 (1C), 115.7 (1C), 115.0 (2C), 42.6 (1C), 33.6 (1C); ESI-MS (M^++H): 300; FAB+ HRMS calcd for $C_{16}H_{15}ON_3\,^{35}Cl$: 300.0904; Found: 300.0902; MS *m/z* 300 (M^++H, 100 %), 235, 217, 192, 180, 176.

Known probe properties: The probe compound is not very soluble in either methylene chloride or deuterated chloroform, but very soluble in dimethyl sulfoxide (DMSO) and somewhat soluble in methanol and ethanol; >90% pure; mp 178-180 °C; ^{1}H NMR (300 MHz, $(CD_3)_2SO$) δ 9.19 (s, 1H), 8.49 (s, 1H), 8.39 (s, 1H), 7.78 (d, 1H, *J* = 9.0 Hz), 7.69 (d, 1H, *J* = 8.7 Hz), 7.05 (d, 2H, *J* = 8.1 Hz), 6.68 (d, 2H, *J* = 8.4 Hz), 3.67 (t, 2H, *J* = 7.5 Hz), 2.83 (t, 2H, *J* = 7.5 Hz); ^{13}C NMR (75 MHz, $(CD_3)_2SO$) δ 159.1 (1C), 156.3 (1C), 156.2 (1C), 148.4 (1C), 133.5 (1C), 130.4 (2C), 130.2 (2C), 130.1 (1C), 122.7 (1C), 116.4 (1C), 115.8 (2C), 43.4 (1C), 34.3 (1C); ESI-MS (M^++H) 300; FAB+ HRMS calcd for $C_{16}H_{15}ON_3\,^{35}Cl$: 300.0904; Found: 300.0902; MS *m/z* 300 (M^++H, 100 %), 235, 217, 192, 180, 176.

Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) and recommendations for the scientific use of probe as research tool: The probe compound is not very soluble in either methylene chloride or deuterated chloroform, but very soluble in dimethyl sulfoxide (DMSO) and somewhat soluble in methanol and ethanol. The compound does not appear to be very reactive. It is not toxic.

Appendices

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