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Compounds blocking mutant huntingtin toxicity identified using a Huntington's disease neuronal cell model

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Neuronal cell death in HD is believed to be largely a dominant cell-autonomous effect of the mutant huntingtin protein. We previously developed an inducible PC12 cell model which expresses an N-terminal huntingtin fragment with an expanded poly Q repeat (N63-148Q) under the control of the tet-off system. In order to evaluate the ability of compounds to protect against mutant huntingtin toxicity in our model, we measured LDH released by dead cells into the medium. We have now screened the library of 1040 compounds from the NINDS Custom Collection as part of a National Institute of Neurological Disorders and Stroke (NINDS) collaborative project. Each positive compound was tested at 3–8 concentrations. Five compounds significantly attenuated mutant huntingtin (htt)-induced LDH release without affecting the expression level of huntingtin and independent of effect on aggregates. We also tested a broad spectrum caspase inhibitor Z-VAD-fmk and previously proposed candidate compounds. This cell model can provide a method to screen potential therapeutic compounds for treating Huntington's disease.

Keywords: Huntingtin; Polyglutamine disease; Compound screen; LDH assay; Cell toxicity; Aggregation

Introduction

Huntington's disease (HD) is a progressive fatal neurodegenerative disorder involving abnormalities of movement, cognition, and emotion (Bates et al., 2002; Ross et al., 1997). It is caused by an expansion of a CAG repeat coding for polyglutamine in the huntingtin gene on chromosome 4 (Huntington's Disease Collaborative Research Group, 1993). Polyglutamine lengths of greater than 36 cause HD, and the length of the expanded polyglutamine repeat is related to the age of onset. The mechanism(s) by which this expanded polyglutamine cause disease remains elusive. There is no currently available treatment which can delay the onset of disease or slow the progression of HD (Grimbergen and Roos, 2003; Hersch, 2003; Hersch and Rosas, 2001; Hughes and Olson, 2001; Jankowsky et al., 2002). Identification of effective compounds that will delay the onset of disease and prevent or attenuate disease progression is a major goal.

At the cellular level, HD is characterized by progressive neuronal death in the striatum, cerebral cortex, and other regions (Bates et al., 2002; Vila and Przedborski, 2003; Vonsattel et al., 1985). Intracellular aggregates consisting of aggregates of misfolded huntingtin protein with other huntingtin interacting molecules are major cellular features in the late stage of HD, though the relationship of aggregation to neuronal death is uncertain (Arrasate et al., 2004; Becher et al., 1998; Bence et al., 2001; Davies et al., 1997; Kuemmerle et al., 1999; Ross and Poirier, 2004; Saudou et al., 1998). In cellular, invertebrate, and mouse models, an N-terminal fragment containing the expanded poly Q is sufficient to cause pathological change including aggregation formation and cell death (Faber et al., 1999; Hickey and Chesselet, 2003a,b; Leavitt et al., 1999; Peters et al., 1999; Sipione and Cattaneo, 2001; Schilling et al., 1999; Mangiarini et al., 1996; Zhou et al., 2003).

The NINDS sponsored an effort to screen a variety of cells and other simple models of neurodegenerative diseases with a library of 1040 FDA-approved drugs selected in association with MicroSource Discovery System (Gaylordsville, CT) (Abbott, 2002; Heemskerk et al., 2002). We developed a cell model expressing a mutant huntingtin N-terminal fragment in an inducible manner with tet-off system in PC12 cells (Igarashi et al., 2003) to screen these 1040 compounds. PC12 cells can be differentiated with NGF and exhibit many characteristics of neurons. Therefore, the system that we developed can be used

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as a tool to screen compounds, and provide support for further preclinical and clinical therapeutic trials of these compounds in HD.

Materials and methods

Chemicals and reagent

Phenyl butyrate, Sodium Butyrate, Congo Red, MK-801, Mithramycin, Depudecin and Trichostatin A, Cystamine dihydrochloride, Staurosporine and 1-methyl-4-phenyl-pyridinium iodide (MPP^+) were purchased from Sigma. Apoptosis inhibitor M50054 (2,2′-methylenebis(1,3-cyclohexanedione)) was purchased from Calbiochem (La Jolla, CA). Suberoylanilide hydroxamic acid (SAHA) was synthesized by ALEXIS Biochemicals.

Plasmid constructs

N-terminal huntingtin fragments containing the first 63 amino acids with 148Q (N63-148Q-myc) or 23Q and myc tag were amplified by PCR using *Sal*I-Kozak consensus sequence-primer (Sal-C-U-ATG primer: GATCGTCGACGCCACCATGGCGACC-CTGGAAAAGCTGAT) and c-*myc* tag-*Hin*dIII primer (N63-*myc*-L-primer: GATCAAGCTTTCAATTCAGATCCT-CTTCTGA-GATGAGTTTTTGTTCCAGCAGCGGCTGTGC-CTGCGG). PCR templates carrying 148Q were used as reported previously (Igarashi et al., 2003; Peters et al., 1999). DNA fragments were sub-cloned into a pTet-splice vector (Life Technologies Inc., Gaithersburg, MD) after digesting with *Sal*I and *Hin*dIII.

Generation of inducible cell lines

Tet-off PC12 cells (Clontech, Palo Alto, CA), stably expressing tTA, were co-transfected with 4 μg of huntingtin construct with 0.2 μg pTK-Hyg (Clontech, Palo Alto, CA) plasmid using Lipofectamine Plus (Life Technologies Inc., Gaithersburg, MD) (Igarashi et al., 2003). The cells were selected in the RPMI 1640 medium (GIBCO) with 5% fetal bovine serum (FBS), 10% horse serum, 100 μg/ml G418, 200 μg/ml hygromycin, 200 ng/ml doxycycline, 100 units/ml penicillin, and 100 units/ml streptomycin. After a 3- to 4-week selection at 37°C in a humidified 5% CO_2 incubator, G418/hygromycin-resistant colonies were isolated and screened for transgene expression by Western blot analysis using a c-*myc* antibody (9E10). We chose line 59 which was highly expressing mutant htt fragment for this study.

Cell culture, differentiation, and induction of transgene expression

Inducible PC12 cell lines were maintained in the RPMI 1640 medium (GIBCO) with 5% Tet system approved fetal bovine serum (Clontech), 10% horse serum (GIBCO), 100 μg/ml G418 (GIBCO), 100 μg/ml hygromycin (Roche), 200 ng/ml doxycycline (Clontech), 100 units/ml penicillin, and 100 units/ml streptomycin (GIBCO). Cells were grown at 37°C in a humidified 6.0% CO_2 incubator and changed to the fresh medium every 48 h. Mutant huntingtin fragment expression was turned on when doxycycline (Dox, a tetracycline derivative) was removed from the culture medium. Cells were differentiated at the differentiation medium [RPMI 1640 medium with 0.5% Tet system approved fetal bovine serum, 1.5% horse serum, 100 μg/ml G418, 100 μg/ml hygromycin, 100 units/ml penicillin and 100 units streptomycin, 50 ng/ml NGF (Roche)]. Expression of mutant htt and cell differentiation were induced at same time, the testing compounds were added to the culture medium once cells were attached to the plate. Cells were plated in collagen-coated 96-well plate (BD Biosciences) at 10^5 cells/ml, doxycycline (200 ng/ml) was added to the differentiation medium to suppress expression of mutant huntingtin for no induction control. Compounds were applied to each 96-well at the final concentration of 10 μM for the initial screening. Cells were grown at 37°C in a humidified 6.0% CO_2 incubator for 4 days.

Western blot analysis

Cells were harvested and collected at the designed time and washed with phosphate-buffered saline (PBS), and lysed with ice-cold lysis buffer (50 mM Tris–HCl pH 7.4, 140 mM KCl, 0.5% Triton X-100, and complete protease inhibitors). Lysis was performed on ice for 30 min. After adding gel loading buffer, lysates were heated at 70°C for 5 min. Cell extracts of N63-148Q were resolved on 4–15% graduated polyacrylamide-SDS gels (BIO RAD) and then transferred into nitrocellulose membranes at 30 V for 6 h. Membranes were blocked for 1 h at room temperature in PBS-0.1%Tween 20 containing 5% skimmed milk. Membranes were then probed with the monoclonal c-*myc* antibody (9E10) at a dilution of 1:2000, for 1 h at room temperature. After washing with 5% milk-PBS, the membranes were incubated with peroxidase conjugated anti-mouse secondary antibody (Boehringer Mannheim) for 1 h at room temperature. Immunoreactive bands were detected by chemiluminescence (Amersham, Buckinghamshire, England).

LDH assay

PC12 cells expressing htt-N63-148Q were treated with different compounds for 4 days. Cytotoxicity Detection Kit (Roche) was used for measurement of lactate dehydrogenase (LDH) released into the medium by dead cells. 25 μl culture medium was transferred to 96-well plates for LDH assay. The LDH working solution was made freshly according to the manufacturer's introduction. 25 μl LDH working solution was added into the 25-μl culture medium and was incubated for 30 min at room temperature. Reaction was stopped by adding 100 μl 0.5 N HCl. Absorbency was read at 490 nm with a plate reader (Molecular Devices Spectra Max 340 PC).

Immunofluorescent cytochemistry and quantization of huntingtin aggregates

For immunofluorescence detection, cells were grown at collagen-coated culture slides (BD Bioscience). Cells were fixed with 4% paraformaldehyde (Ted Pella, Inc.) at the designed time points, then permeabilized with 0.1% Triton X-100 for 5 min; cells were then exposed to primary antibody c-*myc* antibody (1:1000) overnight at 4°C, followed by incubation with Fluorescein (FITC) conjugated anti-mouse IgG (Jackson lab) for 30 min at room temperature. Cells were