GSK-3β inhibitors prevent cellular polyglutamine toxicity caused by the Huntington’s disease mutation

Jenny Carmichael, Katherine L. Sugars, Yi Ping Bao and David C. Rubinsztein*

Department of Medical Genetics, Cambridge Institute for Medical Research, Wellcome/MRC Building, Addenbrooke’s Hospital, Hills Road, Cambridge, CB2 2XY, UK

*Corresponding author: DC Rubinsztein; Phone: 01223 762608; Fax: 01223 331206

EMAIL: DCR1000@CUS.CAM.AC.UK

Running title: GSK-3β inhibition prevents polyglutamine toxicity
SUMMARY

Huntington’s disease (HD) is one of nine known neurodegenerative disorders caused by an expanded polyglutamine (polyQ) tract in the disease protein. These diseases are associated with intraneuronal protein aggregates. Heat-inducible chaperones like HSP70 and HSP27 suppress polyQ aggregation and/or toxicity/cell death. Heat shock transcription factors, including HSF-1, regulate HSP70 and HSP27 expression. HSF-1 activity is reduced by glycogen synthase kinase-3 (GSK-3) and enhanced by GSK-3 inhibitors, like lithium. Thus, we hypothesized that lithium treatment may partially rescue death in HD cell models. LiCl reduced polyQ toxicity in neuronal and non-neuronal cell lines but this was not associated with elevation of HSP70 or HSP27. The protective effect of lithium involved GSK3β inhibition, as polyQ toxicity was also reduced by SB216763, a GSK3β inhibitor, and by overexpression of a dominant-negative GSK3β mutant. LiCl and SB216763 increased β-catenin-dependent tcf-mediated transcription. Since β-catenin overexpression protected cells from polyQ toxicity, we tested if this pathway was impaired by a polyQ expansion mutation. Cells expressing expanded repeats had reduced β-catenin levels associated with a parallel decrease in Tcf-mediated transcription, compared to cells expressing wild-type constructs. Since LiCl can protect against polyglutamine toxicity in cell lines, it is an excellent candidate for further in vivo therapeutic trials.
INTRODUCTION

Huntington’s disease (HD) is a member of a family of neurodegenerative diseases caused by an abnormal CAG expansion in the coding region of the affected gene. Other members of this group include spinocerebellar ataxias (SCA) 1,2,3,6 and 7, dentatorubral-pallidoluysian atrophy (DRPLA) and spinobulbar muscular atrophy (SBMA). The CAG repeats code for an expanded polyglutamine (polyQ) tract that causes disease by conferring a novel gain of function on the mutant protein (1). HD is characterised by expansions of a polyQ stretch in exon 1 of the Huntingtin gene to more than 37 glutamines, and a short N-terminal fragment encoding the polyQ stretch is sufficient to cause aggregates and toxicity in mice (2, 3) and in cell models (e.g. 4, 5). Indeed, many believe that the mutant protein acquires its toxicity and its propensity to aggregate after cleavage, forming a short (so far, incompletely-defined) N-terminal fragment containing the polyglutamine stretch (5).

A pathological hallmark of HD is the formation of intracellular aggregates in specific parts of the brain in affected individuals. The exact role of these aggregates in the pathogenesis of HD is unclear: some studies have suggested that the formation of intracellular aggregates might be directly pathogenic (6-9), but others have argued that aggregates may not be deleterious (10-12). Although it is controversial whether the intraneuronal aggregates are directly pathogenic, it is more widely accepted that polyglutamine diseases are caused by misfolded, aggregate-prone proteins (13).

Cells control the levels of aggregate-prone proteins by a number of mechanisms, including molecular chaperones of the Hsp 40/Hsp 70 families. Indeed, we and others
have shown that overexpression of HSP70 and HSP40 family members reduce both aggregation and cell death in cell models of HD and related conditions (reviewed in 14). We recently found that heat shock protein 27 (HSP27) also suppressed polyQ-mediated cell death. In contrast to HSP40/70 chaperones, we showed that HSP27 suppressed polyQ death without suppressing polyQ aggregation – our results suggested that the poly Q mutation induced reactive oxygen species (ROS) which directly contributed to cell death and that HSP27 acted by reducing ROS levels (14).

The expression of certain heat shock proteins (like HSP27 and HSP70) is induced by various stressors like heat, oxidants and heavy metals. This heat shock response is mediated by heat shock transcription factors (HSFs), including HSF-1. Recently Bijur et al. (15) showed that overexpression of glycogen synthase kinase-3 (GSK-3) reduced the activation of HSF-1 and production of Hsp 70 in response to heat shock; these effects of GSK-3 were partially reversed by treatment with lithium which is known to inhibit GSK-3. Thus, we hypothesised that lithium treatment may enhance heat shock protein levels and partially rescue death in our HD cell models.

GSK-3β is a highly conserved, constitutively active, serine/threonine kinase which has been implicated in many fundamental cellular functions, such as the cell cycle, gene transcription, cytoskeletal integrity and apoptosis, as a result of its ability to phosphorylate key proteins that modulate these processes (16). GSK-3β is ubiquitously expressed, including abundant expression in the brain (17). Some of the proposed target substrates of GSK-3β include transcription factors such as HSF-1, c-jun and c-myc, enzymes which regulate metabolism such as glycogen synthase and microtubule
associated proteins such as tau.

The activity of GSK-3β is subject to regulation by two different types of mechanisms (16). GSK-3β activity can be regulated by phosphorylation, which can be modulated by diverse pathways including insulin signalling, growth factors and amino acids. GSK-3β is active in its non-phosphorylated form and site-specific phosphorylation at Ser⁹ inhibits its activity. The second mechanism for GSK-3β regulation is independent of phosphorylation at Ser⁹ and involves the highly conserved Wingless (Wnt) signalling pathway, which specifies cell fate, proliferation and differentiation in Drosophila, Xenopus and mammals. In the absence of a Wnt signal, unphosphorylated active GSK-3 is present in a multiprotein complex (together with axin and the adenomatous polyposis coli protein – APC) in which GSK-3β phosphorylates β-catenin, thereby targeting it for ubiquitin-mediated degradation (18). In the absence of nuclear β-catenin, transcription factors of the TCF/LEF family (T-cell factor/lymphoid enhancer-binding factor-1) occupy target gene promoters in a complex with various co-repressors to mediate transcriptional repression (19). When Wnt proteins bind to the frizzled receptor on the cell surface, the dishevelled protein is activated via a signalling pathway that has yet to be fully elucidated. This inhibits GSK-3 activity by disrupting the multiprotein complex, which allows β-catenin to be freed, preventing its phosphorylation and degradation (20). The free accumulated cytosolic β-catenin can translocate into the nucleus, where it enhances the transcription of Wnt target genes by binding to transcription factors of the TCF/LEF family (21, 22). The target genes whose increased transcription is responsible for enhancing resistance to apoptosis have yet to be identified.
Recent work using relatively specific GSK-3β inhibitors, such as lithium or a selective small molecule inhibitor called SB216763, has supported the hypothesis that GSK-3β is pro-apoptotic. Overexpression of GSK-3β in SH-SY5Y neuroblastoma cells potentiated apoptosis while lithium and SB216763 were reported to protect SH-SY5Y cells against staurosporine or heat shock-induced apoptosis (15). Part of lithium’s anti-apoptotic properties may be mediated by downregulation of p53 and upregulation of Bcl-2 (23).

Lithium is a safe, well established drug which is the mainstay of treatment of unipolar and bipolar disorders and is used both in the acute phase and as prophylaxis for recurrent manic and depressive episodes. Since GSK3β may be able to regulate polyQ pathogenesis, lithium was an obvious choice to test as a possible pharmacological modulator of polyglutamine induced toxicity, as it inhibits GSK3β.
EXPERIMENTAL PROCEDURES

CELL CULTURE, TRANSFECTION EXPERIMENTS AND FACS ANALYSIS

African green monkey kidney cells (COS-7) and human neuroblastoma cells (SKNSH) were grown in DMEM (Sigma, Dorset/UK) supplemented with 100 IU/ml penicillin/streptomycin, 2mM L-glutamine, 1mM sodium pyruvate and 10% fetal bovine serum (FBS) and maintained at 37°C, 5% CO2 in a humidified incubator.

For experiments the cells were pretreated for 3 days with 2.5 mM or 5 mM LiCl (Sigma, Dorset/UK) or for 24 hours with SB216763 (Glaxo Smith Kline, UK) prior to transfection. Treatment was maintained throughout the experimental period after transfection.

For transfection, cells were seeded in 6 well plates on coverslips and grown to 60-80% confluency for 24 hours. COS-7 cells were exposed to a mixture of 10µl LipofectAMINE Reagent™ (Invitrogen,UK) and 2µg of plasmid DNA for 5 hours in serum free medium, after which full culture medium was added. For efficient transfection SKNSH cells required LipofectAMINE PLUS Reagent ™ (Invitrogen, UK), otherwise the protocol was the same as for COS-7 cells.

In co-transfection experiments, we used HD exon 1 constructs in pEGFP-C1 (described previously in 4); pcDNA3 (Invitrogen, San Diego, CA) as an empty vector control; pcDNA 3.1 constructs expressing wild type β-catenin and β-catenin containing a missense mutation of tyrosine for serine at codon 33 (S33Y) which makes it resistant to degradation (kind gifts from Hans Clevers); and a dominant negative GSK-3 β mutant (kind gift from Geoffrey Cooper). We used a 3:1 ratio of the test construct to HD exon 1.
construct DNA, to ensure that all cells expressing HD exon 1 constructs also expressed the appropriate test constructs. In all such experiments we used a total of 2 µg DNA/3.5 cm dish.

To determine whether LiCl or SB216763 affected levels of EGFP-HDpolyQ expression, COS-7 cells were pretreated with LiCl or SB216763 as described above and cells were transfected with EGFP-HDQ23. Quantitative flow cytometry was performed using a FACSort flow cytometer (Becton Dickinson, Oxford, UK). 10,000 – 20,000 cells per sample were examined and the data were analysed using WinMDI software.

**Analysis of polyglutamine induced aggregation**

We analysed 300-400 EGFP expressing cells with similar fluorescent signal per slide in multiple randomly chosen fields, blinded to the treatment condition. The proportion of EGFP expressing cells with one or more intracellular inclusions was used as a measure of inclusion formation (14, 24, 25).

**Analysis of cell viability**

To assess cell viability we looked at nuclear morphology and EGFP-expressing cells with fragmented or condensed nuclei were counted as dead. Nuclear fragmentation was detected by nucleus staining with 4’,-6-diamidino-2-phenylindole (DAPI) as described previously (4). We have demonstrated that these criteria are specific for cell death, as they show a very high correlation with propidium iodide staining in live cells (14). Furthermore, these nuclear abnormalities are reversed with caspase inhibitors (4, 14, 25). Analysis was performed with the observer blinded to the identity of the slides and all
experiments reported in the figures were done in triplicate at least twice. At 48 hours post transfection, cells on coverslips were washed with 1x PBS, fixed with 4% paraformaldehyde in 1x PBS for 30 min. and mounted in antifadent supplemented with DAPI at 3 µg/ml to allow visualisation of nuclear morphology using fluorescent light microscopy.

**Statistical analysis**

Pooled estimates for the changes in inclusion formation or cell death, resulting from pertubations assessed in multiple experiments were calculated as odds ratios (OR) with 95% confidence intervals [(%cells expressing construct with inclusions in pertubation conditions/ % cells expressing construct without inclusions in pertubation conditions)/ (%cells expressing construct with inclusions in control conditions/ % cells expressing construct without inclusions in control conditions)]. Odds ratios and p values were determined by unconditional logistical regression analysis, using the general log-linear analysis option of SPSS 9 software (SPSS, Chicago). Odds ratios were considered to be the most appropriate summary statistic for reporting multiple independent replicate experiments of this type (4, 14, 25), because the percentage of cells with inclusions under specified conditions can vary between experiments on different days, whereas the relative change in the proportion of cells with inclusions induced by an experimental perturbation is expected to be more consistient.

**Western blotting**

Samples were trypsinised and lysed with 1 x SDS-PAGE Laemmli buffer containing protease inhibitors (Roche, Lewis, UK). The proteins were resolved in 12.5% denaturing
gels and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia) with a semi-dry transfer apparatus (Bio-Rad Laboratories). The membranes were probed with mouse monoclonal antibodies against Hsp 27 and Hsp 70 (Stressgen Biotecnologies, Canada), Bcl-2 and p53 (Diaclon Research, France), a mouse monoclonal anti-β-catenin antibody (Zymed, San Francisco, CA), and a rabbit monoclonal antibody against actin (Sigma, UK). All primary antibodies were used in a concentration of 1:1000, except the anti-β-catenin antibody which was used in a concentration of 1:500. Blots were probed with sheep anti-mouse or donkey anti-rabbit Horseradish Peroxidase linked antibodies (Amersham Pharmacia) in a concentration of 1:2000 and bands were detected using an enhanced chemiluminescence reagent (Amersham Pharmacia) according to the manufacturer’s instructions.

**Immunocytochemistry**

COS-7 cells were grown on coverslips for 16-24h and transfected with GFP-Q74 construct or co-transfected with GFP-Q74 and the β-catenin construct. 48h after transfection, cells were washed and fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.5% Triton X-100 in PBS for 15 min. After three washes with PBS, cells were incubated in blocking buffer (5% FBS) for 30 min. Antibodies were mouse anti-β-catenin (Zymed, San Francisco, CA), rabbit anti-GSK-3β (Stressgen, Victoria BC, Canada). Antibodies were used at 1:600 for 1h at RT and washed in 1xPBS three times. Then cells were incubated with either goat anti-mouse IgG or goat anti-rabbit IgG conjugated to Texas Red (Molecular Probes, Eugene, OR) for 1h in the dark at RT (1:1000). After washed three times in PBS, the cells were mounted in antifadent
containing DAPI. Samples were observed using a confocal microscope.

**β-catenin/Tcf Luciferase assay**

The pTopflash (wild type) and pFopflash (mutant) constructs (Upstate Biotechnologies, NY, USA) contain a luciferase reporter under the control of two repeats each containing three copies of the T-cell factor (Tcf) binding site (wild type) upstream of the thymidine kinase minimal promoter. pFopflash contains the mutated Tcf binding sites and was used as a negative control.

COS-7 and SK-N-SH cells were pre-treated with LiCl or SB216763 as described, seeded in 24 well plates and transfected with 750 ng of pTopflash or pFopflash using LipofectAMINE Reagent™ (Invitrogen, UK) or LipofectAMINE PLUS Reagent™ (Invitrogen, UK) respectively. 300 ng of a β-galactosidase reporter plasmid under the control of a constitutively active human elongation factor 1α promoter was cotransfected in each well to control for variations in transfection efficiency. Cell lysates were harvested ~24 hours after transfection, and levels of luciferase and β-galactosidase activity determined (25). The luciferase activity from each well was normalised to β-galactosidase activity. To measure and normalise the amount of luciferase activity specifically due to the presence of Tcf binding at different doses of LiCl or SB216763, we divided the luciferase activity in cells expressing pFopflash from those expressing pTopflash. Each experiment was conducted twice in triplicate and the results were analysed using linear regression analysis.

Stably-inducible PC12 HD-Q74 (lines 1b and 10) and PC12 HD-Q23 (lines 14 and 20) (25), were transfected as described above. Cells were left uninduced or induced with
1μg/ml doxycycline after the initial 5 hour transfection period. Cells were harvested 48 hours following transfection and reporter assays were done as described above.
RESULTS

Chronic LiCl treatment reduces both polyglutamine-induced aggregation and cell death

We have previously shown that transient or stable induced expression of an EGFP-tagged HD exon1 fragment with 74 glutamines (EGFP-HDQ74) in different cell lines is associated with the formation of intracellular aggregates in a proportion of cells. EGFP-HDQ74 causes significantly more cell death, compared to the corresponding “wild type” EGFP-HDQ23, which does not form aggregates (4,24,25).

We tested the effect of chronic treatment with 2.5 mM or 5mM LiCl on polyglutamine aggregation and cell death in COS-7 (non-neuronal) and SK-N-SH (neuronal) cell lines (Fig. 1). These LiCl concentrations mimic the extracellular fluid levels that would be seen in vivo with doses used to treat patients with bipolar affective disorder (26). We pre-treated cells with LiCl for 3 days prior to transfection and continued treatment after transfection as a number of studies (27,28) have suggested that acute treatment does not have a protective effect against cell death. Cells were analysed 48 hours post transfection, when 40-50% of EGFP-positive untreated/control COS-7 cells expressing EGFP-HDQ74 had inclusions, and 40-45% showed nuclear fragmentation. At this time point 40-47% of control SK-N-SH cells expressing EGFP-HDQ74 contained inclusions and the same proportion of cells had fragmented/condensed nuclei. LiCl treatment showed a consistent and significant protective effect against polyglutamine-induced cell death in both cell lines (Figs. 1a and 1b). LiCl also significantly reduced aggregate formation by EGFP-HDQ74 in both cell lines (Figs. 1c and 1d). Odds ratios (ORs; see methods) were
considered to be the most appropriate summary statistic for multiple independent experiments of this type, because the percentage of cells with nuclear abnormalities under specified conditions varied between experiments on different days, whereas the relative change in the proportion of cells nuclear abnormalities induced by a perturbation is expected to be more constant (4, 14, 24, 25).

**GSK3β inhibition decreases polyglutamine-induced cell death.**

While LiCl is believed to be a relatively specific inhibitor of GSK-3β, it is likely to have other effects (29). Therefore, we tested whether the protective effect of LiCl against polyglutamine-induced cell death was mimicked by the apparently specific GSK-3β inhibitor, SB216763 (30). In both COS-7 and SK-N-SH cells, SB216763 treatment was associated with a dose-dependent reduction in polyglutamine-induced cell death compared to controls (Figs. 2a and 2b). However, in contrast to LiCl, SB216763 was associated with an increase in the proportion of cells with aggregates (Figs. 2c and 2d). To further confirm that GSK-3β inhibition could reduce polyglutamine-induced cell death, we used a dominant negative GSK-3β mutant (31) in a co-transfection experiment together with EGFP-HDQ74 in SK-N-SH cells. As shown in figure 3, the dominant-negative mutant had the same effect as SB216763, and decreased polyglutamine-induced cell death but increased the proportion of cells with aggregates (compared to an empty vector control).

Compared to untreated control cells, neither LiCl nor SB216763 (at the highest concentrations used) were toxic to the cells and neither compound modulated the low
background levels of cell death in the cells expressing EGFP-HDQ23, as assessed by flow cytometry (data not shown; see methods). We examined levels of protein expression by flow cytometry using EGFP-HDQ23, as transient transfection with this construct gives uniform cytoplasmic EGFP expression and does not interfere with the fluorescence read-out signal. In contrast, a proportion of cells expressing the mutant EGFP-HDQ74 constructs form intracellular aggregates which tend to sequester all the cytoplasmic EGFP and result in a spuriously low fluorescence read-out signal. Neither LiCl nor SB216763 (at the highest concentrations used) modulated EGFP-HDQ23 expression.

**LiCl treatment does not mediate its effects by upregulating Hsp70, Hsp 70 or Bcl-2.**

In order to test if LiCl and SB216763 protected against polyglutamine-induced toxicity by upregulating the levels of stress-inducible heat shock proteins, we analysed western blots on COS-7 and SK-N-SH cells, which had been treated with LiCl or with SB216763 according to the protocol that had been used for the EGFP-HDQ74-transfected cells. Neither compound induced any significant changes in the expression levels of Hsp-70 or Hsp-27 (Figs. 4a and 4b).

Since LiCl may mediate anti-apoptotic effects by upregulating Bcl-2, we tested whether overexpression of a Bcl-2 construct together with EGFP-HDQ74 could rescue polyglutamine induced toxicity. We found that Bcl-2 overexpression significantly reduced polyglutamine-induced death in SK-N-SH cells (Fig. 5). However, this mechanism is unlikely to be responsible for the protection mediated by LiCl or SB216763 in our HD model, since neither compound induced detectable changes in the levels of protein expression of Bcl-2 or p53 (which negatively regulates Bcl-2 expression) (Figs.
LiCl activates a pro-survival pathway through GSK-3\(\beta\) inhibition and activation of \(\beta\)-catenin/Tcf–mediated transcription.

To determine whether LiCl had an effect on GSK-3-mediated \(\beta\)-catenin signalling, we used the pTopflash or pFopflash luciferase reporter plasmids. The pTopflash (wild-type) and pFopflash (mutant) constructs contain a luciferase reporter under the control of two repeats each containing three copies of the T-cell factor (Tcf) binding site (wild-type) upstream of the thymidine kinase minimal promoter. pFopflash contains mutated Tcf binding sites and was used to control for the transcriptional activity of the reporter that was independent of Tcf elements. We co-transfected COS-7 cells, pre-treated with LiCl or SB216763, with pTopflash or pFopflash and a \(\beta\)-galactosidase reporter plasmid under the control of a constitutively active human elongation factor 1\(\alpha\) promoter in a 3:1 ratio. \(\beta\)-galactosidase activity was quantified to control for transfection efficiency in each well. In each condition, we compared the wild-type to mutant Tcf-containing vectors to determine the extent of transcriptional activation specifically due to the Tcf elements. LiCl treatment caused a dose-dependent increase in \(\beta\)-catenin-mediated transcription (\(p=0.05, R^2=0.77\)), and this effect was even more pronounced with SB216763 treatment (\(p=0.0004, R^2=0.89\)) (Fig. 6).
To confirm that the protective effect of LiCl against polyglutamine-induced toxicity could be mediated through activation of Tcf transcription, we overexpressed either wild type β-catenin or the S33Y β-catenin mutant which is resistant to degradation (32), together with EGFP-HDQ74 in COS-7 and SK-N-SH cells. As shown in figure 7, overexpression of either wild-type β-catenin or the S33Y β-catenin mutant in COS-7 and SK-N-SH cells decreased polyglutamine-mediated cell death (compared to an empty vector control) but increased the proportion of EGFP-positive cells with inclusions. The trend towards an enhanced protective effect of the S33Y mutant in SK-N-SH cells is compatible with its greater stability.

**β-catenin levels and Tcf-mediated transcription are reduced in cells expressing expanded polyglutamine repeats**

The above data suggest that GSK 3β inhibition rescues polyglutamine-mediated cell death by increasing levels of Tcf-mediated transcription, which is mediated by β-catenin. Thus, we tested if this component of the Wnt signalling pathway was impaired by the HD mutation. In order to avoid the confounds of triple transfection and cell death in reporter gene studies of promoter activity, we studied this pathway in our PC12 stable doxycycline-inducible cell lines (25). These lines, which we have previously characterised, show minimal cell death for the first 3 days after induction (25) and allow us to analyse promoter activity using conventional double transfection with the luciferase reporter gene of interest and β-galactosidase-expressing vector to control for transfection efficiency. We studied two independent mutant and two independent wild-type lines induced to express either EGFP-HDQ74 or EGFP-HDQ23 constructs. Cells were transfected with pTopflash or pFopflash vectors 5h before induction and analysed 48h after
induction. Fig 8 shows that Tcf-mediated transcription was significantly reduced in the HDQ74 lines, compared to the HD-Q23 lines. This reduction was associated with a parallel reduction in β-catenin levels (Fig 9).

A plausible explanation for this effect was that β-catenin may be sequestered to inclusions. However, neither endogenous, nor exogenously transfected β-catenin were associated with nuclear and cytoplasmic inclusions formed by EGFP-HDQ74 in COS-7 cells (data not shown). We also observed no association of GSK3β with inclusions (data not shown).
DISCUSSION

Our data shows that inhibition of GSK3β by LiCl, SB216763 or a dominant negative GSK3β construct protect against polyglutamine-induced death in SK-N-SH and COS-7 cells. These protective effects are mediated, at least in part by β-catenin, since LiCl and SB216763 enhance the β-catenindependent Tcf transcription pathway and β-catenin overexpression protects against polyglutamine-induced death. The protective effect of LiCl at concentrations mimicking what would be seen at therapeutic doses in vivo, suggests that this drug needs serious consideration for therapeutic trials in HD mouse models, which we are planning. However, such trials will need to be carefully conducted given the narrow therapeutic range of this drug.

While all the compounds with GSK3β inhibitory activity reduced cell death caused by the HD mutation, it was interesting that LiCl reduced the proportion of cell with aggregates but SB216763, the dominantnegative GSK3β and β-catenin increased the proportions of cells with aggregates. It is possible that LiCl may perturb other pathways unrelated to GSK3β (29). If GSK3β inhibition modulated cell death but not aggregation, then the apparent dissociation between aggregation and cell death seen with SB216763, the dominantnegative GSK3β and β-catenin would occur if aggregates were protective, epiphenomena or deleterious: aggregate formation will be more likely in cells which remain attached to a coverslip for longer times due to the reduced rate of cell death.

The protective effects of GSK3β inhibition in our model did not appear to be associated with a heat shock response, or due to elevated levels of Bcl-2 and decreased p53. While
some studies have suggested that GSK3β modulates these pathways, Cohen and Frame have argued that many of the putative GSK3β substrates have not yet been shown to fulfil a series of criteria that are needed for them to be considered as true physiological targets (33).

Since overexpression of β-catenin rescued polyglutamine-induced cell death, we tested if this pathway was impaired in our HD cell model. In cells expressing expanded repeats, there was a reduction in β-catenin levels that was associated with a parallel reduction in Tcf-mediated transcription, as compared to cells expressing wild-type constructs. It is unclear how the polyglutamine expansion mutation exerts this effect. Neither β-catenin nor GSK3β were associated with aggregates. It may be difficult to elucidate how the HD mutation affects β-catenin levels, as many of details of the Wnt signalling pathway upstream of GSK3β have yet to be elucidated.

In conclusion, we find that GSK3β inhibition rescues polyglutamine-induced cell death in neuronal and non-neuronal cell lines. This protection is mediated by increases in β-catenin and its associated transcriptional pathway. This pathway is likely to be relevant to the polyglutamine-induced cell death in our models, as we demonstrated decreased levels of β-catenin and Tcf-mediated transcription in cells expressing expanded repeats. Since LiCl, a commonly used drug, can protect against polyglutamine toxicity via this pathway in both neuronal and non-neuronal cell lines, it is an excellent candidate for further in vivo therapeutic trials.
Acknowledgements

WE THANK DRS HANS CLEVERS AND GEOFFREY COOPER FOR KIND GIFTS OF B-CATEN
3B MUTANT CONSTRUCTS AND DAVID HOWLETT AND STEPHEN TROWBRIDGE AT GL:
SB216763. WE ARE GRATEFUL TO THE VIOLET RICHARDS CHARITY, THE ISAAC NEWTC
MUSCULAR DYSTROPHY CAMPAIGN FOR FUNDING. JC IS AN ACTION RESEARCH TRAI
AND IS GRATEFUL FOR A SACKLER STUDENTSHIP AND DCR IS A WELLCOME TRUST SI
CLINICAL SCIENCE.
Figure Legends

Figure 1

**LiCl protects against polyQ-induced cell death and inclusion formation.**

COS-7 (a and c) and SK-N-SH cells (b and d) were pretreated with LiCl for 3 days prior to transfection with EGFP-Q74 and the treatment was continued during the 48 hours of transfection. EGFP-positive cells were scored for inclusion formation and nuclear fragmentation 48 hours after transfection. LiCl treatment consistently and significantly reduced nuclear fragmentation (a and b) and reduced inclusion formation (c and d). The results represent 2 independent experiments each done in triplicate. 300 - 400 cells were counted per slide.

*** - p< 0.0001, ** - p< 0.001

Figure 2

**SB216763 protects against cell death but increases inclusion formation in cells expressing EGFP-HDQ74.**

Cells were pretreated with SB216763 for 24 hours prior to transfection with EGFP-Q74 and the treatment was continued during the 48 hours of transfection. EGFP-positive cells were scored for inclusion formation and nuclear fragmentation 48 hours after transfection. SB216763 treatment significantly reduced nuclear fragmentation in COS-7 (a) and SK-N-SH cells (b) and increased inclusion formation at the same time (c and d). DMSO served as a solvent control for SB216763. The results represent 2 independent experiments each done in triplicate. 300 - 400 cells were counted per slide.

*** - p< 0.0001, ** - p< 0.001, NS - not significant
Figure 3

A dominant-negative GSK-3β mutant reduces poly-Q induced cell death

In SK-N-SH cells transfected with EGFP-Q74, overexpression of a dominant negative GSK-3β mutant significantly reduced cell death as evidenced by nuclear fragmentation (a) and increased inclusion formation (b). The figure represents the odds ratios for 2 independent experiments each done in triplicate. The error bars represent the 95% confidence interval for the odds ratio. *** - p< 0.0001

Figure 4.

LiCl or SB216763 do not modulate expression of Hsp-70, Hsp-27, Bcl-2 or p53.

Western blots showing no detectable up-regulation in Hsp-70 (a), Hsp-27 (b), Bcl-2 (c) or p53 (d) expression in SKNSH cells treated with LiCl for 5 days or SB216763 for 72 hours. These periods mimic what would be seen after a 48 h transfection, taking the pre-treatment into account (see methods). All blots were probed with anti-actin antibody as a control for loading.

Lane 1 - control cells without any treatment
Lane 2 - cells treated with 2.5 mM LiCl
Lane 3 - cells treated with 5 mM LiCl
Lane 4 - cells treated with 2.5μM SB216763
Lane 5 - cells treated with 5 μM SB216763
Lane 6 - cells treated with 10 μM SB216763
Lane 7 - cells treated with DMSO as a solvent control
Lane 8 - SKNSH cells subjected to heat shock as a positive control
Lane 9 - COS-7 cells subjected to heat shock as a positive control
Lane 10 (panel C) - SKNSH cells overexpressing Bcl-2 as a positive control

Neither LiCl nor SB216763 caused any upregulation of Hsp70, Hsp27 or Bcl-2 in similar western blot experiments in COS-7 cells (data not shown). While Hsp27 was detectable in COS-7 without heat shock (data not shown) (in contrast to SK-N-SH cells (b)) and was induced by heat shock in these lines (b), we observed no obvious Hsp27 signal nor induction in SK-N-SH cells (b). Cells were heat shocked at 42°C for 30 minutes, 24 after seeding and cells were harvested for analysis 48 h after heat shock.

Figure 5

**Bcl-2 overexpression protects against polyQ toxicity**

In SK-N-SH cells transfected with EGFP-Q74, overexpression of a Bcl-2 vector reduced cell death as evidenced by nuclear fragmentation (a) and increased inclusion formation (b). Cells were analysed 48 h after transfection. The figure represents the odds ratios for 2 independent experiments each done in triplicate. The error bars represent the 95% confidence interval for the odds ratio.

*** - p< 0.0001

Figure 6

**LiCl and SB216763 activate Tcf-dependent transcription in COS-7 cells.**

COS-7 cells were pre-treated with LiCl for 3 days or SB216763 for 24 hours prior to transfection with either pTOPflash or pFOPflash luciferase reporter plasmids, and the treatment was continued during the 24 hours post transfection. β-galactosidase reporter
plasmid was cotransfected to normalise for transfection efficiency. Luciferase activity was measured 24 hours post transfection. The fold activation was determined by comparing pTOPflash activity with pFOPflash activity and the results are expressed as a ratio of pTOPflash: pFOPflash. Two experiments using the different doses were performed in triplicate and each point represents the mean of the activation values from a triplicate experiment. Results were analysed using linear regression analysis. For LiCl treatment p= 0.05, R²=0.77; For SB216763 p= 0.0004, R²=0.89

Figure 7

β-catenin protects against polyQ toxicity

COS-7 cells and SK-N-SH cells were transfected with wild type β-catenin or S33Y β-catenin mutant and EGFP-HDQ74 in 3:1 ratio. Cells were scored for inclusion formation and nuclear fragmentation 48 hours after transfection. Overexpression of both wild-type β-catenin or S33Y β-catenin mutant consistently and significantly reduced nuclear fragmentation in both cell lines (a and c) and significantly increased inclusion formation in both COS-7 (b) and SKNSH cells (d). The results represent 2 independent experiments each done in triplicate. 300 - 400 cells were counted per slide. *** - p< 0.0001, ** - p< 0.001.

Figure 8.

Tcf-mediated transcription is reduced in PC12 stable inducible cell lines expressing EGFP-HDQ74, compared to EGFP-HDQ23.

Data are shown for 2 independent clonal lines with each repeat length (EGFP-HDQ74.1b, EGFP-HDQ74.10, EGFP-HDQ23.14 and EGFP-HDQ23.10) induced for 48
h. Specific Tcf-mediated luciferase activity was determined by comparing pTOPflash activity with pFOPflash activity and the results are expressed as a ratio of pTOPflash:pFOPflash. Data represent means and SEM of two experiments each in triplicate. Similar significant trends were observed if we subtracted pFOPflash from pTOPflash activities.

Figure 9

β-catenin levels are reduced in cells expressing expanded polyQ repeats

Western blot showing downregulation of endogenous β-catenin in PC 12 cells expressing EGFP-HDQ74 (lanes 1 and 2) as compared to cells expressing EGFP-HDQ23 (lanes 3 and 4). Data are shown for two independent clonal lines for both EGFP-HDQ74 and EGFP-HDQ23, each induced for 48 hours. The blot was re-probed with anti-actin antibody as a control for loading. This result was consistently reproducible.

Lane 1 – EGFP-HDQ74.10
Lane 2 - EGFP-HDQ74.1b
Lane 3 - EGFP-HDQ23.20
Lane 4 - EGFP-HDQ23.14
References


a  Nuclear fragmentation in SK-N-SH cells

b  Inclusion formation in SK-N-SH cells
a  Nuclear fragmentation in SKNSH cells

![Graph showing nuclear fragmentation in SKNSH cells.](image)

- Odds ratios with 95% CI
- pCDNA 3 vs. Bcl-2

b  Inclusion formation in SKNSH cells

![Graph showing inclusion formation in SKNSH cells.](image)

- Odds ratios with 95% CI
- pCDNA 3 vs. Bcl-2
GSK-3β inhibitors prevent cellular polyglutamine toxicity caused by the Huntington's disease mutation

Jenny Carmichael, Katherine L. Sugars, YiPing Bao and David C. Rubinsztein

*J. Biol. Chem. published online July 3, 2002*

Access the most updated version of this article at doi: 10.1074/jbc.M204861200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2002/07/03/jbc.M204861200.citation.full.html#ref-list-1