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Research Report

PBT2 Reduces Toxicity in a C. elegans Model of polyQ Aggregation and Extends Lifespan, Reduces Striatal Atrophy and Improves Motor Performance in the R6/2 Mouse Model of Huntington's Disease

Robert A. Cherny[a,b,*], Scott Ayton[a], David I. Finkelstein[a,b], Ashley I. Bush[a], Gawain McColl[a] and Stephen M. Massa[c]
[a]*The Mental Health Research Institute, Kenneth Myer Bldg, The University of Melbourne, Royal Pde, Parkville, VIC, Australia*
[b]*Prana Biotechnology Ltd., 369 Royal Pde, Parkville, VIC, Australia*
[c]*Department of Neurology, Department of Veterans Affairs Medical Center and University of California, San Francisco, CA, USA*

Abstract.
Background: There is evidence that interaction with biologically important metals, particularly copper and iron, contributes to the pathological aggregation and toxicity of the mutant *huntingtin* protein in HD. *PBT2* is a novel 8-hydroxyquinoline drug in clinical trials for AD and HD which restores metal homeostasis and has demonstrated neuroprotective and cognitive benefits in animal models of AD and in a Phase IIa clinical trial in AD patients.
Objectives: We assessed efficacy of PBT2 to improve function in nematode and mouse models of HD.
Methods: We assessed the effects of PBT2 on motility in a strain of *C. elegans* engineered to overexpress an extended polyglutamine tract. We then assessed the effects of daily oral administration of PBT2 on the R6/2 mouse a vertebrate model of HD. Behavioural measures (Rotarod performance, clasping latency and duration) and measures of overall health (body weight, lifespan) were assessed throughout the study. At conclusion brains were evaluated for weight and lateral ventricle area.
Results: PBT2 significantly decreased paralysis caused by overexpression of an extended polyglutamine tract in a *C. elegans*. R6/2 mice treated with PBT2 showed significantly better performance in the Rotarod task and significantly decreased duration of clasping than control animals. Mean body weight and brain weight in the PBT2 treatment group were significantly higher than the control cohort and median lifespan was prolonged by 26%. Mean lateral ventricle area in the PBT2-treated group was 46% smaller than in the control group.
Conclusions: Treatment with PBT2 may be beneficial as a disease modifying therapy for HD.

Keywords: PBT2, C. elegans, R6/2, metals

*Correspondence to: Robert Cherny PhD, The Mental Health Research Institute, The University of Melbourne, Royal Pde, Parkville, VIC, Australia. Tel.: +61 3 90356714; E-mail: rcherny@mhri.edu.au.

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INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurological disorder that causes progressive cognitive, motor, and psychiatric dysfunction over a course of 10 to 20-years, leading to death. HD is caused by CAG-repeat expansion in the 5' region of the *IT15* gene that encodes the ubiquitously expressed 350-kDa protein huntingtin (Htt). The expanded CAG region encodes polyglutamine (polyQ). When the polyQ region is 40 residues or more, there is virtually 100% penetrance of the disease phenotype [1, 2]

Current therapies for Huntington's disease (HD) provide some symptomatic relief but do not target the underlying pathology and do not slow disease progression. Across the spectrum of neurodegenerative diseases there is evidence of failure of homeostatic mechanisms regulating the trafficking and localisation of vital biological metals, (primarily copper zinc and iron). Under this milieu the increasingly labile metal pool can interact promiscuously with cellular components, promoting oxidative damage and exacerbating the pathological aggregation and toxicity of susceptible proteins including *beta amyloid (Abeta*) in Alzheimer's disease (AD) [3, 4] and mutant *Huntingtin* protein (mHtt) in HD [5–7]. We have been investigating the therapeutic potential of the 8-hydroxyquinoline chemical class which is characterised by redox-silent metal binding, moderate affinity for transition metals and ability to readily traverse the blood brain barrier. When bound to either copper or zinc the resulting drug-metal complex undergoes a conformational change from a charged to a neutral hydrophobic species favouring the facile translocation of the metal across the neuronal plasma membrane in a manner reminscent of an an ionophore or physiological metal chaperone. Notably, a recent, unbiased screening of approximately 200,000 compounds identified the 8-hydroxyquinoline chemical class as the most effective at preventing toxicity caused by the pathological aggregation of misfolded proteins including polyglutamine [8]. In a proof of concept study we previously showed [9] that the 8-hydroxyquinoline compound clioquinol (CQ) reduced striatal atrophy and produced motor benefits in an animal model of HD, simultaneously restoring defective neuronal metal homeostasis [4, 10–13] and reducing mHtt protein accumulation. *PBT2* is a novel, orally bioavailable 8-hydroxyquinoline metal chaperone in clinical development for AD and HD which has demonstrated neuroprotective and cognitive benefits in animal models of AD [10, 14, 15] and significant improvements in executive function in a Phase IIa clinical trial in AD patients [16–18]. We investigated the effect of PBT2 on aspects of motor function and viability in two transgenic animal models of HD: The nematode *C. elegans* which expresses an extended poly Q tract and the R6/2 mouse, in which Exon 1 of the human HD gene is overexpressed.

MATERIALS AND METHODS

C. elegans: AM140 (*rmIs132*[P*unc-54*::Q35-YFP]) [19] was obtained from the Caenorhabditis Genetics Center. Cultures were maintained at 20°C on Nematode Growth Media (NGM) [20] with *E. coli* (strain OP50). PBT-2 (Prana Biotechnology Ltd, Australia) was dissolved in ethanol (<1 ml) and added to molten NGM (at 55°C) at a final concentration of 10 μg/ml. Bacteriological activity was suppressed in all media (control and compound) by the addition of 50 μg/ml Ampicillin (Sigma). Media was stored at 4°C and used within one-week. Developmentally synchronized *C. elegans* cultures were transferred onto NGM ± PBT2 as L4 larvae (48 h post egg lay) for 24 h at 20°C, and then transferred and maintained at 25°C as young adults (time zero). Populations were then scored for paralysis each day as previously described [19]. Nematodes were scored as paralysed if they failed to complete a spontaneously or touch-provoked head to tail body bend.

Statistics: The proportion of individuals not paralysed was determined and confidence intervals calculated [21]. Comparisons at different ages were made using a 2-proportion, 2-tailed Z-test.

The R6/2 mouse model of Huntington's disease

Mice transgenic for exon 1 of the human HD gene with a greatly expanded CAG repeat recapitulate many of the features of human HD. They develop progressive behavioral deficits, characterized by hindlimb clasping on tail suspension, impaired performance on motor tests and changes in activity, as well as weight loss, striatal atrophy reflected in increased size of the lateral ventricles, reduced hippocampal neurogenesis [22], cognitive decline and premature death. The appearance of protein aggregates precedes symptoms and is found throughout the brain post-mortem. The R6/2 mouse has an accelerated phenotype, with onset of motor symptoms beginning at 5–6 weeks, progressing to a severe impairment at 12–14 weeks [23]. To obtain test populations, first, wild type (B6CBAF1/J) males were bred with wild type females transplanted with HDexon1/+ (R6/2) ovaries (Jackson Laboratory, Bar Harbour,

Maine, USA). R6/2 heterozygote males obtained from this cross were then bred with B6CBAF1/J females to obtain groups of heterozygote and wild-type males, collected from the 3rd through 6th generations. Genotyping was performed as previously described [24].

PBT2 treatment

PBT2 in standard suspension vehicle (SSV [25], was administered to mice by oral gavage from 3 weeks of age at a dose of 30 mg/kg/day. This dose was selected as it produced significant behavioural and neurobiological improvements in an APP/PS1 AD mouse model [10]. Treatment groups were: R6/2 + PBT2 ($n = 10$), R6/2 + SSV ($n = 10$), w/t + PBT2 ($n = 12$), w/t + SSV ($n = 12$).

Statistical methods used in the animal study

Behaviour of animals in each treatment group at each week of testing was analysed by *t* test. Life span time was analysed by Kaplan-Meier survival curves. Lateral ventricular area was analysed by 2-way Anova. Statistical tests were performed using SPSS software.

Note on statistical approach: The analysis of R6/2 mice is complicated by their premature death. This being the case, approaching the conclusion of the study, animals remaining at each weekly testing interval will be enriched with the longest living animals, which presumably are the healthiest of the group. This will artificially alter the mean health outcomes, favoring a healthier phenotype. The imputing of data did not manifestly alter the outcome of any assay; indeed the imputed data frequently generated a more conservative outcome.

Behavioral tests

Researchers were blind to treatment during experimental testing and data collection. A single investigator conducted each behavioral test. All procedures were performed in accordance with protocols approved by the San Francisco Veterans Administration Medical Center Animal Care Committee.

Rotarod testing

Rotarod performance was assessed as described in [24] using the SDI Rota-Rod (San Diego Instruments, San Diego) with rotation accelerating to 40 rpm over 4 minutes. Testing was performed at 4, 8, 10 and 12 weeks of age. The latency to falling from the rod was recorded for 3 trials/session. When a mouse died prior to testing, it was allocated 8 sec, the lowest recorded latency to fall.

Clasping

Foot clasping, a dystonic posturing of the hind limbs on suspension by the tail is characteristic of the later stages of disease in R6/2 mice and is typical of striatal damage [26]. To assess this behaviour, mice were suspended by the tail for 30 seconds, and the foot-clasping time was recorded. The time taken for clasping to commence (t[sec], latency) was also recorded. Clasping observations were conducted once per week from 4 weeks of age and daily during the period of drug treatment (as per [9]). To minimize experimenter variability, a single individual performed all tests. Mice that did not perform the clasping were allocated 30 seconds for latency (the best recorded score) and 0 seconds for duration. Mice that died were allocated 0 seconds for latency and 30 seconds for duration (the worst possible score).

Body weight and lifespan

Body weight was measured once per week, starting at 1 week of age. All mice were observed daily in order to determine lifespan. Mice were euthanized if found moribund, as defined by lack of movement even after prodding, and/or lying on side with lack of righting reflex. After a mouse died, its last recorded weight was imputed for the remaining analysis. This ensured that we did not bias our results toward the healthiest mice in the study.

Brain weight and anatomy

At 10 weeks, mice were anesthetized and transcardially perfused with PBS followed by 4% paraformaldehyde/PBS. Perfused mouse brains ($n = 6$ for each treatment group) were weighed, fixed in 4% paraformaldehyde for a further 1 h at room temperature and stored in 30% sucrose overnight at 4°C. Brain tissue was snap frozen, then cryosectioned at 40 μm thickness using a Leica CM1850 cryostat (Leica, Germany). Sections were then stained with haematoxylin and eosin. Each section through the lateral verticals was analyzed for ventricular area; areas of each section were pooled to produce a total ventricular area for each animal.

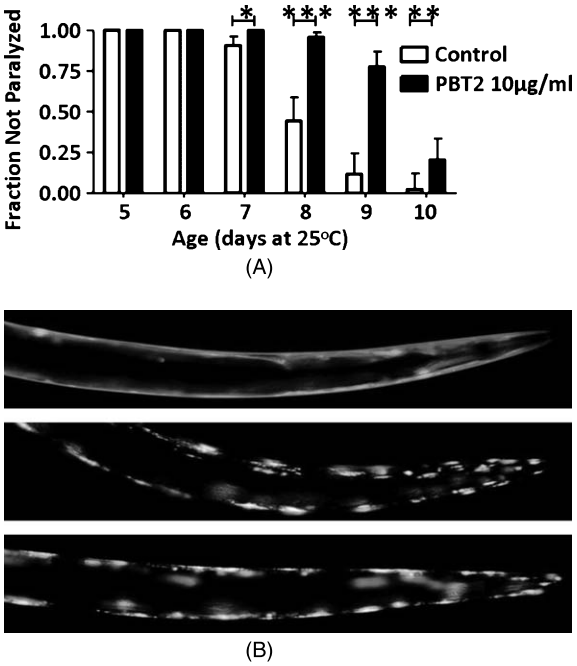

Fig. 1. PBT2 treatment reduces paralysis in a *C. elegans* model of poly-Q overexpression. Paralysis associated with poly-Q expression is protected by PBT2. A. Plotted are the proportions of individuals not paralysed with upper and lower 95% confidence intervals, Shown are poly-Q animals with PBT2 (PBT2,10µg/ml, $n=43$) and without (Control, $n=49$), where $n=$ number of individuals assayed. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. One of two independent experiments is shown. B. Representative live epi-fluorescence imaging of *C. elegans* expressing Q35::YFP. 4-day-old adults (Top) show diffuse Q35::YFP distribution, whilst 8-day-old treated with (Bottom) and without (Middle) 10 µm/ml PBT showed similar *in vivo* aggregation of Q35::YFP.

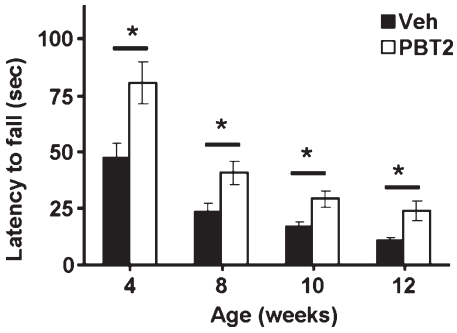

Fig. 2. PBT2 treatment improves Rotarod performance of R6/2 mice. R6/2 mice and w/t controls ($n=10$ per group) were treated with 30 mg/kg PBT2 or vehicle and tested for their ability to perform the Rotarod task at 4, 8, 10 and 12 weeks of age. Mice treated with PBT2 remained longer on the Rotarod at weeks 4, 8 10 and 12 (*t* test, $p=0.012$, 0.02, 0.012 and 0.011 respectively). $^{*}p<0.05$ Error bars represent mean ± SEM.

RESULTS

We found that PBT2 significantly delayed the onset of paralysis in a *C. elegans* model of polyQ over-expression [27]. In this model, expression of 35-glutamine repeats fused to Yellow Fluorescent Protein (YFP) results in an age-dependent transition of the polyQ from diffuse cytoplasmic to aggregated. A progressive paralysis phenotype is associated with this aggregation of polyQ. Interestingly, the ability of PBT2 to ameliorate the paralysis phenotype in the *C. elegans* model was not associated with a reduction in aggregated polyQ-YFP fluorescence (data not shown), supporting the proposition by Lajoie et al. (2010) that the coalescing polyQ precipitates may be less relevant pathologically than the soluble oligomeric species. Significant differences were observed in Rotarod performance of PBT2 treated R6/2 mice compared with vehicle treated littermates at 4 (*T*-test, $p=0.012$), ($p=0.02$), 10 ($p=0.012$) and 12 ($p=0.011$) weeks of age (Fig. 2). PBT2 had no effect on performance in wild type mice (data not shown). Clasping is a behavioral phenotype of R6/2 associated with striatal degeneration. Latency to clasp (Fig. 3A) was attenuated with PBT2 treatment at week 7 ($p=0.048$) and week 10 ($p=0.009$). Clasping duration (Fig. 3B) was reduced in the PBT2 treated R6/2 mice in week 8 ($p=0.036$), week 10 ($p=0.002$), week 11 ($p=0.006$), week 12 ($p=0.006$), and week 14 ($p=0.019$). R6/2 mice typically do not gain weight normally as they age and lose weight in the later stages of the disease. The body weights of PBT2 treated R6/2 mice diverged significantly from that of the untreated mice from week 7 onwards as determined by *t*-tests (Fig. 4). Week 7 ($p=0.04$), 8 ($p=0.013$), 9 ($p=0.007$), 10 ($p=0.005$), 11 ($p=0.005$) 12 ($p=0.011$) and 13 ($p=0.010$). The median lifespan of PBT2-treated R6/2 mice (Fig. 5) was 26% greater than that of vehicle treated transgenic littermates ($p=0.003$). The lateral ventricles are enlarged in R6/2 mice, reflecting striatal atrophy. Lateral ventricle area in PBT2 treated mice ($n=4$) was reduced compared with vehicle treated mice ($p=0.001$) (Fig. 6). The mean brain weight of the PBT2-treatment arm ($n=5$) was also significantly higher than that of the vehicle-only R/2 mice ($p=0.026$) (Fig. 7).

DISCUSSION

Metals are essential to normal neuronal function and are highly abundant in the brain relative to other tissues.

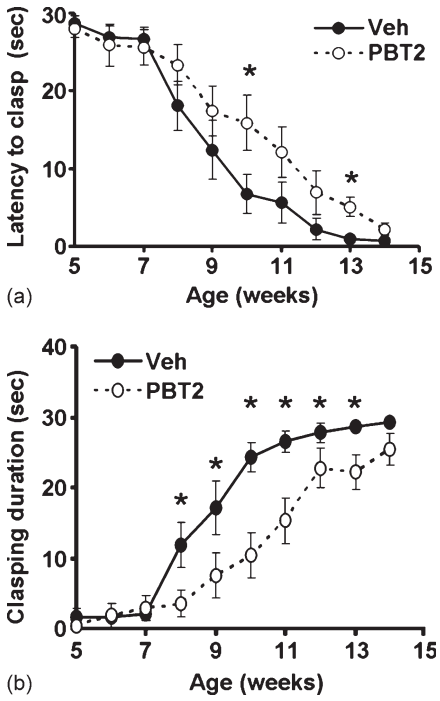

Fig. 3. PBT2 treatment reduces clasping behaviour of R6/2 mice. R6/2 mice exhibit a characteristic dystonic clasping of the hindlimbs. PBT2 treated R6/2 mice ($n = 10$) exhibited significantly less clasping behaviour than vehicle treated animals (Veh) ($n = 10$) at the time points indicated (mean ± SEM.) *T*-tests show significant differences in latency to clasping (Fig. 3a) and duration of clasping (Fig. 3b) at the weeks indicated. $*p < 0.05$.

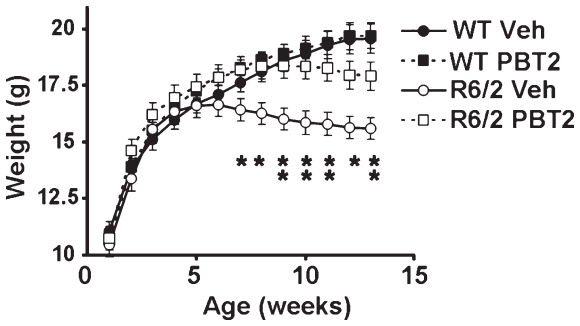

Fig. 4. PBT2-treated R6/2 mice maintain body weight. The body weights of all animals in the four experimental arms were measured every week ($n = 9$ per group). Values for each group at each week were compared using Student's *t* test. The body weights of PBT2-treated R6/2 mice were significantly higher than untreated animals at the weeks indicated by asterisks. $*p < 0.05$, $**p < 0.01$. Error bars represent mean ± SEM.

One consequence of this abundance is the preponderance of neurological disorders in which aberrant metal homeostasis has been identified. In particular, metal-mediated oxidative processes have been implicated

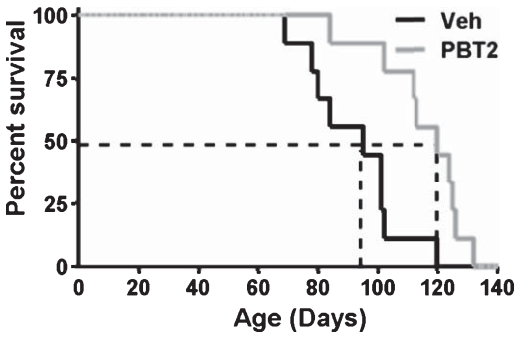

Fig. 5. PBT2-prolongs survival of R6/2 mice. Median survival for the PBT2 group ($n = 10$) was 26% longer than that of the vehicle treated group (Veh) ($n = 10$) (Kaplan-Meier, $p = 0.003$). Mantel-Cox test used for pair-wise comparison. Dashed line X axis intercepts represent median age at death for each group.

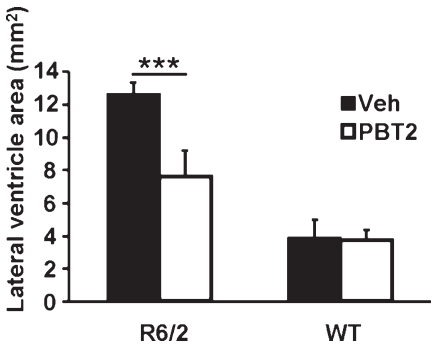

Fig. 6. PBT2 reduces lateral ventricle area in R6/2 mice. Brains of animals from each group ($n = 4$) were sectioned and the area of the lateral ventricles (LV) was measured. LV area of PBT2-treated R6/2 mice was significantly smaller than that of the vehicle-treated Tg littermates (***t-test, $p < 0.001$). Error bars represent mean ± SEM.

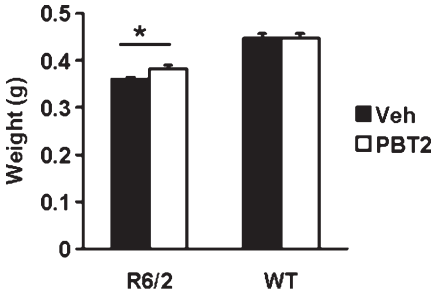

Fig. 7. PBT2 reduces brain weight decline in R6/2 mice. Brains of animals from each group ($n = 5$) were extracted and weighed. The reduction in brain weight associated with R6/2 mice was attenuated with PBT2 treatment (*t-test, $p < 0.05$) Error bars represent mean ± SEM.

in the neurotoxic cascade in HD and other neurodegenerative conditions characterized by pathological protein aggregation including Alzheimer's disease,

Parkinson's disease and Motor Neuron Disease [10, 28, 29]. Clinical evidence of a role for metals in HD has recently been provided using an advanced magnetic resonance imaging technique [7]. In their study Rosas et al. observed that elevated iron levels in specific brain regions, notably the basal ganglia and cortex, correlated closely with CAG triplet repeat number and disease severity and also predated and predicted age of onset. In the R6/2 mouse we previously observed potent motor and neuroprotective effects of the 8-hydroxyquinoline compound CQ, accompanied by significant reduction in levels of aggregated mHtt [9]. The presumed mechanism of action via metal binding was supported by Fox et al., [30] in which they described a role for copper in the pathophysiology of HD which was recapitulated in the R6/2 mouse. Significantly, CQ was shown to inhibit the copper mediated aggregation of mHtt *in vitro* and *in vivo*. More recently, we reported that the N-terminal oligomeric fragments of mHtt (N171-40Q) that are abundant in a knock-in CAG140 HD mouse line readily aggregate in the presence of copper in a redox dependent fashion and that consequent oxidative modification of the cysteines impedes proteolytic breakdown and slows clearance from the soluble protein pool [6]. PBT2 is being developed as a potentially disease modifying drug for AD and HD (Prana Biotechnology Ltd). In a Phase IIa clinical trial in moderate AD patients [16–18], patients receiving the highest dose of 250 mg/day showed a highly significant improvement in executive function associated with significantly reduced levels of beta-amyloid protein in the cerebrospinal fluid. Improved cognition with PBT2 in animal models has now been shown to correlate strongly with elevated markers of neuronal function and plasticity including NMDAr, TrKb, proBDNF, BDNF, CAMkII, spinophillin and synaptophysin as well as significant increases in hippocampal dendritic spine density [14]. *In vitro* studies using cultured primary neurons described in the same report showed that the ability of PBT2 to influence these neuroanatomical and biochemical substrates of memory and learning was strictly dependent on the presence of Cu and/or Zn in the growth medium, the beneficial effects abolished by the presence of a powerful metal chelator. A further study [31] has revealed that these beneficial changes may be effected via pathways which involve inhibitory phosphorylation of glycogen synthase kinase 3β (GSK3β). Elevating cellular Zn levels with PBT2 also promoted phosphorylation of cAMP response element binding (CREB), activating phosphorylation of CaMKII and inhibiting caspase-3. Thus the therapeutic effects of metal chaperones like PBT2 are thought to be due to intervention in multiple pathological events resulting from metal dyshomeostasis including inhibition of metal mediated oxidative damage, intervention in the aggregation process and restoration of neuronal function.

In the current study we found that PBT2 significantly delayed the onset of poly-Q-induced paralysis in *C. elegans*, which has been used as a model for polyglutamine repeat diseases including HD. *C. elegans* has been promoted as a rapid system to screen drugs for their potential to prevent toxicity caused by misfolded proteins in a multi cellular animal [32, 33]. In this model the poly-Q is expressed in the cytoplasm of bodywall muscle cells where it undergoes an age-dependent transition from soluble to aggregated. This transition correlates with a fully penetrant paralysis phenotype. It is widely held that the toxic species of the so-called protein misfolding diseases are forms of diffusible oligomers that exist in a dynamic equilibrium with the precipitable, histologically detectable forms [34]. Evidence for the presence of this type of toxic soluble oligomer has also been reported for mHtt [35]. We have shown that PBT2 can prevent the formation of soluble oligomers of Aβ [10] and in an independent study PBT2 was reported to prevent suppression of long term potentiation in murine hippocampal slices exposed to a preparation of soluble oligomeric Aβ species [36]. Interestingly, the ability of PBT2 to ameliorate the paralysis phenotype in the *C. elegans* model was not associated with a reduction in fluorescence (Fig 1B), supporting the proposition that the coalescing polyQ precipitates may be less relevant pathologically than the putative soluble oligomeric species.

In the R6/2 mouse model of HD we found that PBT2-treated animals showed significantly superior motor performance in the Rotarod task with reduced clasping behaviour compared with vehicle-treated animals. These differences were sustained over the lifetime of the animal. These indices of improved behaviour and health were accompanied by a significant increase in mean brain and body weight and a reduction in lateral ventricular volume, suggesting a significant attenuation of the striatal atrophy that characterises this mouse model. The 26% prolongation of median lifespan in the PBT2 treatment group was particularly meaningful given the aggressive phenotype in this animal model and compares favourably with the 20% extension of lifespan achieved using CQ. Age-matched non-transgenic littermates were unaffected in any parameter examined by the administration of PBT2, confirming our previous finding that the beneficial effects of the drug are not due to some non-specific

stimulatory mechanism. This study was limited to evaluation of the therapeutic potential of PBT2, as circumstances did not permit the investigation of markers associated with the presumed mechanism of action. This notwithstanding, the substantial body of evidence from our extensive studies of PBT2 in AD models and CQ would suggest a combination of redox silencing of a labile metal pool, detoxification of aggregated protein fragments and/or inhibition of further aggregation, coupled with neuronal repair resulting from the metal chaperone action of PBT2. A separate study dedicated to investigating the mechanism of action of PBT2 in an HD mouse model and specifically its effects upon different soluble, insoluble and oligomneric populations of mHtt is planned.

CONCLUSION

No disease-modifying treatment for HD is available. Psychiatric symptoms are treated to modest effect with the same antipsychotic and antidepression drugs used in the general population, while the motor symptoms are treated with neuroleptics, benzodiazapines or antiparkinsonian drugs. In 2008, despite some significant side effects, the legacy compound Tetrabenazine was approved by the US FDA as the only drug specifically to treat the Huntington's chorea. Tetrabenazine is believed to act as a vesicular monoamine transporter (VMAT), promoting the clearance of dopamine. There is no effective treatment for the cognitive impairment associated with HD. Since becoming widely available as a model for HD the R6/2 mouse has generated numerous reports describing the preclinical efficacy of potential treatments (reviewed in [37]. These include inhibition of protein misfolding [26], transglutaminase inhibition [38], rescue of energy deficits [39] and the restoration of neurogenesis [40]. In terms of prolonging lifespan, of the 60 small molecule interventions cited in a recent review [37] the effect of PBT2 is exceeded only by the chemotherapy drug mithramycin, believed to act by opposing mutant Huntingtin inhibition of gene expression [41] and a combination of the NMDA antagonist Remacemide and the antioxidant coenzyme Q10 [42]. Coenzyme Q10 which appears to have accounted for the majority of the therapeutic benefit, was not effective in a 6 month clinical trial [43] although it is being re-evaluated in a Phase III 5 year trial sponsored by the Huntington Study Group (clinicaltrials.gov). At this point mithramycin has not been trialed in HD patients, likely due to safety concerns with chronic dosing. PBT2 has shown preclinical efficacy in animal models of AD and the predictive value of these models borne out in a Phase IIa clinical trial. We have shown here that PBT2 can protect against poly-Q overexpression in *C. elegans* and confers signficant motor and neuroprotective benefits in the R6/2 mouse model. Thus PBT2 has the potential to influence both cognitive and motor manifestations of neurodegenerative diseases involving misfolded proteins. A Phase II clinical trial of PBT2 in HD patients is currently underway (Clinicaltrials.gov identifier: NCT01590888).

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