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B2 Attenuates Polyglutamine-Expanded Androgen Receptor Toxicity in Cell and Fly Models of Spinal and Bulbar Muscular Atrophy

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Expanded polyglutamine tracts cause neurodegeneration through a toxic gain-of-function mechanism. Generation of inclusions is a common feature of polyglutamine diseases and other protein misfolding disorders. Inclusion formation is likely to be a defensive response of the cell to the presence of unfolded protein. Recently, the compound B2 has been shown to increase inclusion formation and decrease toxicity of polyglutamine-expanded huntingtin in cultured cells. We explored the effect of B2 on spinal and bulbar muscular atrophy (SBMA). SBMA is caused by expansion of polyglutamine in the androgen receptor (AR) and is characterized by the loss of motor neurons in the brainstem and spinal cord. We found that B2 increases the deposition of mutant AR into nuclear inclusions, without altering the ligand-induced aggregation, expression, or subcellular distribution of the mutant protein. The effect of B2 on inclusions was associated with a decrease in AR transactivation function. We show that B2 reduces mutant AR toxicity in cell and fly models of SBMA, further supporting the idea that accumulation of polyglutamine-expanded protein into inclusions is protective. Our findings suggest B2 as a novel approach to therapy for SBMA.

Key words: polyglutamine disease; SBMA; androgen receptor; B2; inclusions

Polyglutamine diseases are late-onset, inherited neurodegenerative diseases caused by expansion of CAG repeats encoding polyglutamine tracts in nine different genes (Orr and Zoghbi, 2007). Expansion of polyglutamine in the androgen receptor (AR), huntingtin, atrophin 1, and ataxin-1, -2, -3, -6, -7, and -17 causes spinal and bulbar muscular atrophy (SBMA), Huntington's disease, dentatorubral-pallidoluysian atrophy, and six types of spinocerebellar ataxia, respectively. All the polyglutamine diseases are inherited in an autosomal dominant fashion, except for SBMA, which is X-linked (La Spada et al., 1991) and gender-specific (Katsuno et al., 2002; Schmidt et al., 2002; Yu et al., 2006). There is no available effective therapy for SBMA and the other polyglutamine diseases, although several therapeutic approaches have been proposed to date (for review see Pennuto and Fischbeck, 2010). Polyglutamine diseases share several features, such as a positive correlation between repeat length and disease severity and the phenomenon of genetic anticipation, which causes the next generation to inherit a longer repeat than the previous one, and so to have an earlier age of onset or a more severe phenotype. Expansion of polyglutamine confers a toxic gain of function on the mutant protein. Evidence also indicates a contribution of loss of protein function to the disease pathogenesis (Zuccato et al., 2001; Thomas et al., 2006;

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Lim et al., 2008). Although disease-specific features imply the contribution of protein-specific features in polyglutamine disease pathogenesis, the observation that the same mutation in nine unrelated genes causes neurodegeneration suggests a common disease mechanism.

Expansion of polyglutamine leads the protein to acquire a stable, non-native β-sheet conformation (Perutz et al., 1994), which results in protein unfolding and deposition into microaggregates and inclusions (Ross and Poirier, 2004). Microaggregates are small oligomeric species detectable by biochemistry (Taylor et al., 2003; Li et al., 2007; Palazzolo et al., 2009). Inclusions are large macromolecular species detectable primarily by immunohistochemistry (Taylor et al., 2003). Inclusions contain several cellular constituents, including molecular chaperones and components of the ubiquitin-proteasome system and autophagic degradation (Garcia-Mata et al., 1999; Wigley et al., 1999; Taylor et al., 2003). The observation that polyglutamine-positive inclusions are present in the nuclei of the degenerating neurons from patients and animal models of polyglutamine diseases led to the idea that inclusions are toxic species (for review see Taylor et al., 2002). Toxicity was attributed to sequestration of essential cellular constituents and aberrant protein–protein interactions, with consequent disruption of cellular homeostasis (Gidalevitz et al., 2006). However, a series of findings has not only dissociated inclusions from neurodegeneration (Cummings et al., 1999; Saudou et al., 1998; Slow et al., 2005; Rub et al., 2006) but also highlighted a protective role for inclusions in neurodegenerative diseases (Taylor et al., 2003; Arrasate et al., 2004). These findings suggest that enhancing inclusion formation may be a therapeutic target for polyglutamine diseases. The compound B2 (5-[4-(4-chlorobenzoyl)-1-piperazinyl]-8-nitroquinoline) has been shown to increase inclusion formation and reduce the toxicity of mutant huntingtin in vitro (Bodner et al., 2006).

Here we investigated the effect of B2 on SBMA. We show that B2 increases formation of mutant AR-positive nuclear inclusions, without altering mutant AR ligand-dependent aggregation, expression, or subcellular localization. The effect of B2 on inclusions correlates with a reduction of AR transactivation, which is not due to altered ligand binding. Finally, we show that B2 reduces the toxicity of mutant AR in both cell and fly models of SBMA. Our results provide evidence that B2 reduces the toxicity of mutant AR by increasing the deposition of the protein into inclusions and highlight B2 as a potential therapy for SBMA.

MATERIALS AND METHODS

Plasmids

The pCMV-AR65Q-K632A,K633A and pARE-E1b-luc expression vectors were kindly provided by Drs. A. Lieberman (University of Michigan, Ann Arbor, MI) and C. Smith (Baylor College of Medicine, Huston, TX), respectively; pFHRE-luc reporter vector was purchased from Addgene.

Cell Cultures and Transfections

HEK293T (ATCC, CRL-1573) and PC12-TET ON cells stably expressing AR112Q (Walcott and Merry, 2002) were cultured as previously described (Walcott and Merry, 2002; Palazzolo et al., 2007). HEK293T cells (6×10^5) were transiently transfected with 1 μg DNA using Lipofectamine Plus (Invitrogen, Carlsbad, CA). PC12-AR112Q cells (8×10^5) were cultured on collagen-coated dishes for 24 hr in differentiation medium (1% heat-inactivated horse serum, 5% heat-inactivated charcoal-stripped fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 132 μg/ml G-418, 70 μg/ml hygromycin B, and 100 ng/μl nerve growth factor) in the presence of doxycycline (10 μg/μl; Calbiochem, La Jolla, CA) and treated with B2 (3448-6548; ChemDiv, San Diego, CA) and R1881 (Sigma, St. Louis, MO) at the indicated concentrations. Motor neuron-derived MN-1 cells stably expressing AR65Q were previously described (Brooks et al., 1997). The cells were maintained in culture in the presence of G418 (350 μg/ml), plated (1×10^6 cells) in charcoal-dextran-stripped fetal bovine serum (HyClone, Logan, UT)-containing medium for 48 hr and processed for caspase 3 assay. Where indicated, the cells were treated with staurosporin (1 μM) for 6 hr and z-VAD-FMK (30 μM) for 48 hr.

Immunocytochemistry and Microscopy

PC12 cells were grown for 24 hr on collagen-coated dishes in differentiation medium, induced for 4 hr with doxycycline, pretreated for 20 hr with B2 (10 μM), and then treated for 48 hr with R1881 (10 nM) and B2. Immunofluorescence was performed as previously described (Palazzolo et al., 2007). The person who analyzed the images was blind for the treatments. For the graph in Figure 1A, the cells treated with R1881 together with either vehicle or B2 were classified into cells with diffuse nuclear AR or cells with nuclear inclusions. The percentage of cells with nuclear inclusions was calculated for each treatment. Data in the graph represent the fold increase in the number of cells with nuclear inclusions in the B2/R1881-treated sample compared with the R1881-treated sample, which was set as 1. The graph represents the average of four independent experiments; in each experiment, three different fields (n = 150 cells) for each treatment were analyzed.

Western Blotting and Nuclear/Cytoplasmic Fractionation

For Western blotting, cells were washed in ice-cold 1× PBS and scraped in lysis buffer [150 mM NaCl, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 5 mM ethylenediaminetetraacetic acid, 1% Na-deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)] plus protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The lysate was sonicated and then centrifuged at 18,000*g* for 10 min at 4°C. Cell lysates were denatured at 95°C in 5× sample buffer (60 mM Tris, pH 6.8, 2% SDS, 25% glycerol, 0.1% bromophenol blue, 20% β-mercaptoethanol) and processed for 7.5–10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a PVDF membrane (Millipore, Bedford, MA).

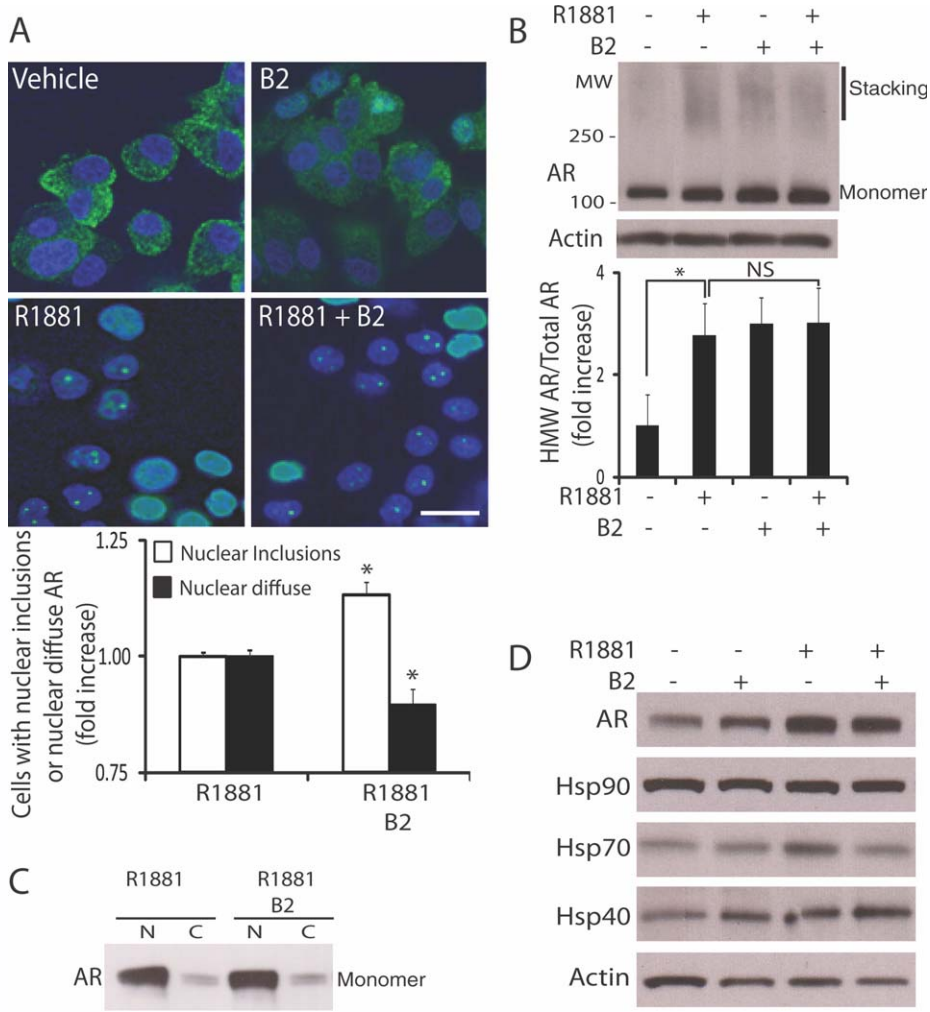

Fig. 1. B2 increases the accumulation of mutant AR into nuclear inclusions. **A:** PC12 cells stably expressing mutant AR (AR112Q) were induced with doxycycline: treated with vehicle, B2 (72 hr), and R1881 (48 hr) as indicated: and processed for immunocytochemistry. AR was detected with N20 antibody (green) and nuclei with DAPI (blue). Quantification of the number of cells with AR-positive nuclear inclusions and of cells with nuclear diffused AR is shown at the bottom. Graph, mean ± sem, n = 4, $\star P = 0.002$ (post hoc *t*-test). **B:** Upper panel: Western blotting of PC12-AR112Q cells showing AR protein in cells treated with R1881 and B2 as indicated in A. Actin is shown as loading control. Shown is one experiment representative of three. MW, molecular weight. Bottom panel: Quantification of mutant AR aggregation reveals that B2 increases AR aggregation in the absence of ligand but has no effect on aggregation in the presence of ligand. HMW, high molecular weight. Graph, mean ± sem, n = 3, $\star P = 0.02$; NS, non-significant (post hoc *t*-test). **C:** Nuclear-cytoplasmic fractionation of HEK293T cells transiently transfected with vector expressing AR65Q and treated as indicated shows that B2 does not affect nuclear translocation induced by ligand. Shown is one experiment representative of three. N, nuclear fraction; C, cytosolic fraction. **D:** Western blotting analysis of PC12-AR112Q cells treated as described for A shows that B2 treatment does not change the expression levels of Hsp90, Hsp70, or Hsp40. Actin is shown as loading control. This is one experiment representative of three. Scale bar = 10 μm.

Immunoblottting was done in 5% nonfat dry milk in Tris-buffered saline. Antibodies used were anti-AR (N20; sc-816; Santa Cruz Biotechnology, Santa Cruz, CA), anti-α-tubulin (T6199; Sigma), anti-Hsp90 (SPA-830; Assay Design), anti-Hsp40 (SPA-400; Assay Design), anti-Hsp70 (Spa810; Assay Design), and anti-actin (sc-1616; Santa Cruz Biotechnology). Immunoreactivity was detected using peroxidase-conjugated AffiniPure goat anti-rabbit or anti-mouse IgG (Jackson Immunoresearch, West Grove, PA), and visualized using Lightning chemiluminescence reagent (Perkin Elmer, Norwalk, CT), following the manufacturer's instructions. Nuclear/cytosolic fractionation was performed per manufacturer's instructions (NE-PER; Pierce, Rockford, IL).

XTT, Caspase 3, Ligand Binding, and Transcriptional Assays

Caspase 3 activity, cell survival (XTT assay), and transcriptional activity were measured according to manufacturers' instructions using the ApoTarget fluorometric assay (Biosource International, Camarillo, CA), Cell Proliferation Kit II (Roche Diagnostics), and LucLite Luminescence Reporter Gene Assay

System (Perkin Elmer), respectively. For the XTT assay, the cells were incubated in aphidicolin (0.4 μg/ml; Calbiochem) to inhibit cell proliferation. For transcriptional assay to measure AR transactivation, cells were transfected with pARE-E1b-luc reporter vector, pre-treated for 24 hr with B2 at the indicated concentrations, and incubated with the ligand together with either B2 or vehicle for other 24 hr. For FOXO-mediated transcriptional activity, cells were transfected with pFHRE-luc reporter vector, pre-treated with B2 for 24 hr, and incubated with IGF-1 or under serum-deprivation conditions for 24 hr prior to the assay. To normalize for transfection efficiency, luciferase activity was compared with β-galactosidase activity. For the ligand binding assay, 24 hr after transfection, HEK293T cells were incubated for 2 hr in 0.5% bovine serum albumin and 10 μM triamcinolone acetonide in Dulbecco's modified Eagle's medium (binding medium) with 10 nM [^{3}H]R1881 (72.0 Ci/mmol; Perkin Elmer). Specific binding of [^{3}H]R1881 was calculated as previously described (Palazzolo et al., 2007).

SBMA Flies

Generation of transgenic flies expressing AR52Q was previously described (Pandey et al., 2007). *Drosophila* stocks were crossed on standard cornmeal agar media at 29°C. GMR-GAL4 virgin females were mated to UAS-AR52Q male flies on media containing either 1 mM dihydrotestosterone (DHT) + 0.5% DMSO or 1 mM DHT + 50 μM B2 diluted in DMSO. The phenotype of female flies was assessed blindly on day 1 post-eclosion. The scoring method was modified from Pandey et al. (2007), as follows: 1 point for presence of bristle phenotype (supernumerary interommatidial bristles or abnormal bristle orientation), 1 point for presence of ommatidial phenotype (fusion or disorganization), 1 point for ommatidial pitting, 3 points for retinal collapse, 3 points if the phenotype covered more than 20% of the eye, 6 points if the phenotype covered more than 50% of the eye. The number of flies analyzed was n = 45 for DHT + vehicle and n = 57 for DHT + B2.

Statistical Analysis

Each experiment was repeated a minimum of three times. One-way ANOVAs were used to evaluate the effect of B2 and ligand among treatment groups. Two-sample *t*-tests were used for post hoc comparisons.

RESULTS

B2 Increases the Formation of AR-Positive Nuclear Inclusions in Cultured Cells

B2 increases the deposition of mutant huntingtin into inclusions (Bodner et al., 2006). We asked whether B2 has similar effect on mutant AR. Mutant AR accumulates into nuclear inclusions in motor neurons in patients (Katsuno et al., 2006). Generation of mutant AR-positive nuclear inclusions with features similar to those observed in patient tissues can be reproduced in an inducible PC12 cell line, which expresses human full-length AR with 112 glutamine residues (PC12-AR112Q; Walcott and Merry, 2002). In these cells, transgene AR expression is induced by treatment of the cells with doxycycline, and inclusion formation is promoted by exposure of the cells to androgens (Walcott and Merry, 2002). To test whether B2 affects inclusion formation in SBMA, the PC12-AR112Q cells were treated with doxycycline and the synthetic androgen analog R1881 together with either vehicle or B2, and the AR-positive inclusions were detected by immunocytochemistry using the AR-specific antibody N20 (Fig. 1A). In the absence of ligand, mutant AR localizes in the cytosol. Treatment of the cells with ligand resulted in nuclear translocation and formation of AR-positive nuclear inclusions. Treatment of the cells with B2 did not induce AR inclusion formation in the absence of ligand. Instead, treatment of the cells with B2 in the presence of ligand significantly increased the number of cells with nuclear inclusions by 13% compared with the cells exposed to ligand alone. Expansion of polyglutamine leads the mutant protein to form not only inclusions but also microaggregates (Taylor et al., 2002; Ross and Poirier, 2004). Mutant AR-positive microaggregates can be detected by Western blotting as high-molecular-weight species accumulating in the stacking portion of polyacrylamide gels (Palazzolo et al., 2009). Therefore, we asked whether B2 affects mutant AR aggregation in the PC12-AR112Q cells (Fig. 1B). Treatment of the cells with ligand significantly increased AR aggregation. Treatment of the cells with B2 increased mutant AR aggregation in the absence of ligand but did not affect the biochemical aggregation of mutant AR induced by ligand.

To investigate whether the effect of B2 on AR inclusions is due to an increase in the AR expression levels, we analyzed the levels of human AR mRNA (Supp. Info. Fig. 1A) and protein (Fig. 1B). By real-time PCR and Western blotting analyses, we found that neither the levels of transgene AR transcript nor the levels of monomeric mutant AR protein change upon B2 treatment. Then, we asked whether B2 increases nuclear inclusion formation because it increases the translocation of mutant AR into the nucleus. To test this, we performed nuclear/cytosolic fractionation in HEK293T cells transiently transfected with vector expressing mutant AR with 65 glutamine residues (AR65Q) and treated with R1881 together with either vehicle or B2 (Fig. 1C). B2 did not change the amount of AR accumulating in the nucleus in the presence of ligand. Because AR interacts with the heat shock proteins (Hsps; Poletti, 2004), we asked whether the effect of B2 occurs through induction of Hsp90, Hsp70, and Hsp40 (Fig. 1D). Expression of these proteins was analyzed by Western blotting in the PC12-AR112Q cells. Treatment of the cells with B2 did not alter the levels of expression of Hsp90, Hsp70, and Hsp40 in either the absence or the presence of ligand, indicating that the B2 effect on AR inclusion is independent of the Hsps. Collectively, these results indicate that B2 increases the deposition of mutant AR into nuclear inclusions without affecting AR ligand-dependent

aggregation, expression, or subcellular distribution in cultured cells.

B2 Alters AR Function

AR is a transcription factor activated by androgens (Poletti, 2004). We reasoned that B2 by entrapping mutant AR into macromolecular complexes may alter the ability of AR to activate transcription. We tested this hypothesis in HEK293T cells transiently transfected with an expression vector encoding AR65Q together with a reporter vector in which luciferase expression is driven by a regulatory region containing three androgen-responsive elements (ARE), as previously described (Palazzolo et al., 2007). The cells were treated with vehicle or B2 (10 μM) and increasing amounts of R1881 (from 0.1 to 10 nM), and AR transactivation was measured by luciferase assay (Fig. 2A). Treatment of the cells with R1881 induced mutant AR transactivation in a dose-dependent fashion. Treatment of the cells with B2 significantly reduced the AR transactivation induced by ligand. As with the effect of B2 on AR inclusions observed in the PC12-AR112Q cells, the effect of B2 on mutant AR transcription observed in HEK293T cells was not due to a decrease in transgene mRNA transcript or protein levels (Supp. Info. Fig. 1B). To investigate whether the effect of B2 also occurs on non-expanded AR and is dose-dependent, we treated cells transfected with either normal or mutant AR with a constant dose of R1881 (10 nM) and a range of concentrations of B2 from 1 μM to 10 μM (Fig. 2B). The effect of B2 on polyglutamine AR transactivation was dose-dependent. At 1 μM, B2 slightly increased normal, but not mutant, AR transactivation, whereas, at 5 μM, B2 had no significant effect on either normal or mutant AR transcription. At 10 μM, B2 reduced both normal and mutant AR transactivation. Similar results were obtained with B21 (Supp. Info. Fig. 2), which is a compound structurally similar to B2 and that has been shown to have the same effect as B2 on mutant huntingtin inclusion formation (Bodner et al., 2006). It is relevant to note that B21 had no effect on normal AR transactivation.

One explanation for the effect of B2 on AR transactivation is the recruitment of AR into inclusions. However, we explored other possibilities. To rule out the possibility that treatment of the cells with B2 disrupts the cellular transcription machinery, we tested whether B2 alters the transcription mediated by FKHRL1, a member of the Forkhead family of transcription factors (Brunet et al., 1999). The Forkhead transcription factors are active in the absence of survival factors, a condition that we reproduced here by serum deprivation, and are inactivated by the insulin-like growth factor 1 (IGF-1) through Akt phosphorylation. HEK293T cells were transfected with a reporter vector in which the luciferase gene is under the control of the forkhead-responsive element (pFHRE-luc; Holtz-Heppelmann et al., 1998). As expected, transcription of the reporter gene was observed upon serum starvation and was decreased by

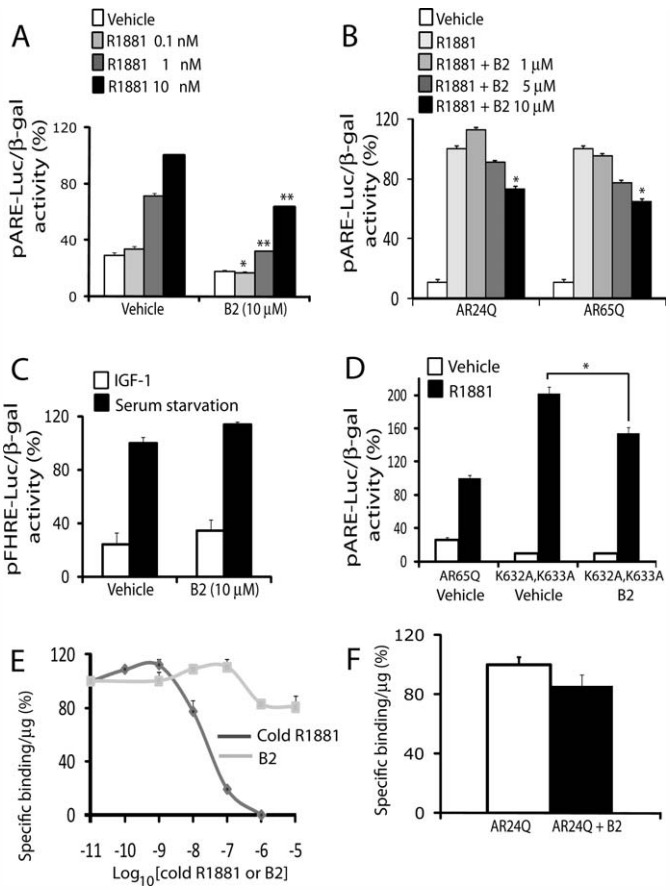

Fig. 2. B2 alters AR transactivation. **A,B:** Transcriptional assay of HEK293T cells transfected with vectors expressing AR65Q (A) or as indicated (B) and the reporter vectors pARE-E1b-luc and pCMVβ for luciferase and β-galactosidase expression, respectively, and treated with B2 (48 hr) and R1881 (24 hr) shows that B2 reduces mutant AR transactivation. Data are represented relative to AR65Q-expressing cells treated with 10 nM R1881, which are set as 100%. Graphs, mean ± sem, n = 3 independent experiments; A: $\star P = 0.05$ and $\star\star P = 0.001$; B: R1881 10 nM, $\star P = 0.004$ (post hoc *t*-test). **C:** Transcriptional assay of HEK293T cells transfected with the pFHRE-luc and pCMVβ reporter vectors, treated with B2 (48 hr), and either serum starved or treated with IGF-1 for 24 hr revealed that B2 does not affect pFHRE reporter activity. Data were analyzed as described for A. Graph, mean ± sem, n = 3. **D:** Transcriptional assay of HEK293T cells transfected with the AR expression vectors indicated and the reporter vectors as for A shows that B2 is active on the acetylation-defective AR mutant. Data were analyzed as in A. Graph, mean ± sem, n = 3, $\star P = 0.02$ (post hoc *t*-test). **E:** Ligand binding assay of HEK293T cells transfected with vector expressing AR65Q, treated with radioactive ligand for 2 hr, then treated with either B2 or cold ligand for 1 hr, shows that B2 does not compete with ligand for binding to mutant AR. Schatchard analysis shows that B2 does not compete for binding with radioactive ligand. Graph, mean ± sem, n = 3 independent experiments. **F:** Ligand binding assay of HEK293T cells transiently expressing normal AR and treated with either vehicle (AR24Q) or 10 μM B2 (AR24Q + B2) shows that B2 does not alter binding of normal AR to ligand. Graph, mean ± sem, n = 3 independent experiments.

IGF-1 treatment (Fig. 2C). Treatment of the cells with B2 did not have any effect on this reporter. These results indicate that B2 does not alter the cellular transcription machinery. Moreover, these data suggest that the effect of B2 on transcription is likely to be specific to mutant AR.

B2 has recently been shown to have inhibitory activity against sirtuin 2 (SIRT2) microtubule deacetylase (Outeiro et al., 2007). Normal AR is acetylated at specific lysine residues lying in the acetylation consensus site KXKK at position 630–633 (NM_000044), where K is lysine and X any amino acid (Fu et al., 2000). To test whether B2 reduces AR transactivation through acetylation at this site, we used an acetylation-defective mutant AR in which the lysine residues were replaced by alanine (AR65Q-K632A,K633A; Thomas et al., 2004). In HEK293T cells, the acetylation-defective mutant had enhanced transactivation compared with the non-substituted AR (Fig. 2D). Similar results were obtained with the non-polyglutamine-expanded AR (Supp. Info. Fig. 3). This is consistent with previous results obtained with non-polyglutamine-expanded AR but different promoter regions (Haelens et al., 2007; Faus and Haendler, 2008). B2 treatment decreased the transactivation of the acetylation-defective mutant, indicating that B2 effect on AR transactivation does not occur through regulation of acetylation at lysines 632 and 633.

Because AR transactivation is strikingly ligand dependent (Poletti, 2004), we wondered whether B2 works as a competitive antagonist. To test this, we incubated HEK293T cells transiently expressing AR65Q cells with nonmetabolizable radioactive ligand [^{3}H]R1881 and measured the displacement of radioactive bound R1881 by increasing amounts of B2 (Fig. 2E). If B2 competes with R1881 for binding to the same site, incubation of the cells with increasing concentrations of B2 is expected to result in a dose-dependent displacement of radioactive ligand from the AR. As positive control, we treated the cells with increasing amounts of cold R1881. B2 did not displace the bound [^{3}H]R1881, whereas cold R1881 completely displaced [^{3}H]R1881 at 100 nM and 1 μM, as expected. These data indicate that B2 does not compete for binding to the same site on the mutant AR where androgens bind, thereby excluding the possibility that B2 acts as a competitive antagonist of AR. At high doses, B2 inhibits normal AR transactivation (Fig. 2B). To rule out the possibility that B2 alters binding of normal AR to ligand, we measured ligand binding in HEK293T cells transfected with normal AR as previously described (Palazzolo et al., 2007). We found that B2 does not alter the binding of ligand to normal AR (Fig. 2F). Altogether, these results show that B2 specifically decreases mutant AR transactivation in a manner that is independent of acetylation at the KXKK site and does not affect ligand binding. Rather, these results are consistent with the idea that the B2-induced formation of AR-positive inclusions results in a reduction of AR function.

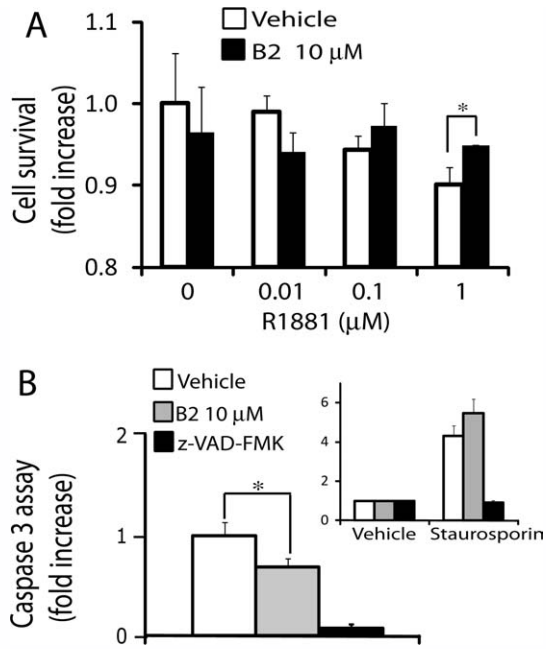

Fig. 3. B2 reduces the toxicity of mutant AR in cultured cells. **A:** XTT assay of PC12-AR112Q cells treated with R1881 (48 hr) together with either B2 or vehicle (72 hr) shows that cell survival is decreased by ligand and that this effect is attenuated by B2. Graph, mean ± sem, n = 3, $\star P = 0.05$ (post hoc *t*-test). **B:** Caspase 3 assay of MN-1 cells stably expressing AR65Q and treated as indicated for 48 hr shows that B2 decreases caspase 3 activity but has no effect on the caspase 3 activation induced by staurosporin (**inset**). The caspase inhibitor z-VAD-FMK (10 μM, 48 hr treatment) and the caspase activator staurosporin (1 μM, 6 hr treatment) were used as controls. Graph, mean ± sem, n = 3, $\star P = 0.004$ (post hoc *t*-test).

B2 Reduces the Toxicity of Mutant AR in Cultured Cells

Because increased accumulation of polyglutamine-expanded proteins into inclusions has been correlated with reduced toxicity in both cell and animal models of polyglutamine disease (Taylor et al., 2003; Arrasate et al., 2004), we asked whether B2 attenuates the toxicity of mutant AR. We tested this in the PC12-AR112Q cells. The cells were treated with R1881 and either vehicle or B2, and cell viability was measured by XTT assay (Fig. 3A). Treatment of the cells with increasing concentrations of R1881 resulted in a dose-dependent decrease in cell viability (Fig. 3A, open bars). Treatment of the cells with B2 significantly increased cell viability by 29% compared with the cells treated with ligand alone (Fig. 3A, solid bars). Similar results were obtained measuring cell death by propidium iodide incorporation (Supp. Info. Fig. 4A).

SBMA is characterized by the loss of lower motor neurons from spinal cord and brainstem (Adachi et al., 2007). Therefore, we asked whether B2 has any effect on mutant AR toxicity in motor neuron-derived MN-1 cells stably expressing polyglutamine-expanded AR with

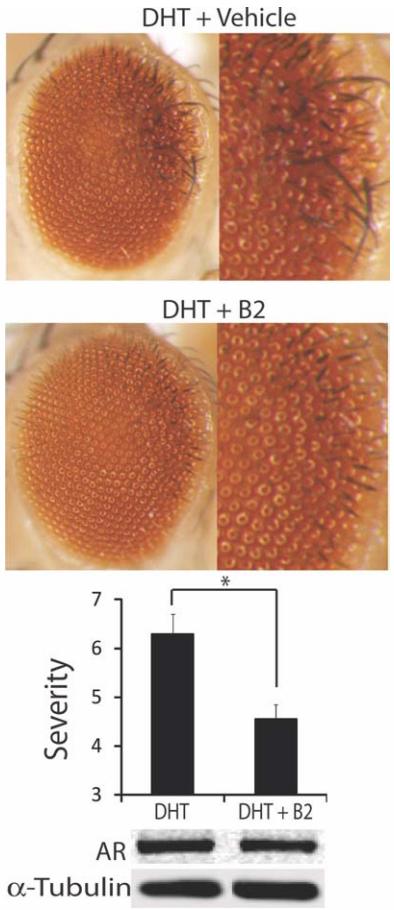

Fig. 4. B2 attenuates the toxicity of mutant AR in vivo. **Upper panel:** Transgenic flies expressing AR52Q in the eye were fed with dihydrotestosterone (DHT) and either vehicle or B2. Exposure of the flies to DHT resulted in the alteration of the eye phenotype, which was attenuated by B2. A magnification of the posterior side of the eye is shown on the right of each panel. **Middle panel:** Quantification of disease severity is shown in the graph (see Materials and Methods). Graph, mean ± sem, n = 45 for the DHT-fed flies and 57 for the DHT/B2-fed flies, $\star P = 0.001$ (two-sample *t*-test). **Bottom panel:** Western blotting analysis of AR transgene expression levels reveals that B2 does not change mutant AR expression in flies. Tubulin (Tub) is shown as loading control.

65 glutamine residues (AR65Q; Brooks et al., 1997). Although these cells do not show ligand-dependent toxicity, they do show polyglutamine length-dependent toxicity. Indeed, we have previously shown that expression of polyglutamine-expanded AR in these cells results in increased caspase 3 activity and reduced cell viability compared with cells expressing non-polyglutamine-expanded AR (Palazzolo et al., 2007). Treatment of the mutant MN-1 cells with B2 reduced caspase 3 activation by 31% (Fig. 3B). The effect of B2 on toxicity was specific to polyglutamine-dependent caspase 3 activation, as B2 did not have any effect on caspase 3 activation induced by staurosporin (Fig. 3, inset). We also asked whether the B2 analog compound B21 impacts the toxicity of mutant AR in the MN-1 cells. Similarly to B2, B21 reduced the caspase 3 activation induced by mutant AR (Supp. Info. Fig. 4B). These results indicate that B2 attenuates the toxicity of mutant AR in cell cultures.

B2 Protects Flies From the Toxicity Induced by Mutant AR

Next, we sought to determine whether B2 counteracts mutant AR-induced neurodegeneration in vivo. With this aim, we used transgenic flies that express mutant AR with 52 glutamine residues (AR52Q; Takeyama et al., 2002; Pandey et al., 2007). Flies expressing polyglutamine-expanded AR recapitulate the unique feature of SBMA, which is the ligand dependence of the disease (Pandey et al., 2007). Transgenic flies expressing AR52Q in the eye do not show any sign of neurodegeneration in the absence of hormone (Pandey et al., 2007). In contrast, the flies show alteration of the eye phenotype when fed with the AR natural ligand dihydrotestosterone (DHT; Fig. 4). Exposure of the flies to B2 together with ligand attenuated the extent of damage. To quantify the effect of B2 on disease severity, we analyzed the phenotype of about 50 flies per group and scored the disease severity as described in Materials and Methods (Fig. 4, middle panel). We found that B2 treatment significantly decreased the extent of alteration of the eye phenotype in this fly model of SBMA. The effect of B2 was not due to a decrease in the level of expression of the mutant AR (Fig. 4, bottom panel). This is the first evidence that B2 protects against polyglutamine-expanded toxicity in vivo. These results are important, because they highlight B2 as a novel potential therapy for SBMA.

DISCUSSION

The current study tested the effect of B2 on SBMA. We found that B2 increases deposition of mutant AR into inclusions. This was associated with reduced transactivation of mutant AR. Furthermore, we show that B2 reduces the toxicity of mutant AR in cell models of the disease. We show for the first time that B2 attenuates polyglutamine-expanded toxicity in vivo. Our results provide further evidence that inclusions represent a protective response of the cell to cope with misfolded protein. Moreover, because we found that the increased accumulation of mutant AR into inclusions correlates with decreased AR function, we speculate that B2 attenuates polyglutamine-expanded toxicity through a mechanism that involves compartmentalization of the mutant protein and reduction of native protein function. Finally, we propose B2 as a potential therapy for SBMA.

B2 Increases the Compartmentalization of Mutant AR Into Inclusions and Reduces Toxicity

B2 was isolated from a drug screen to increase the formation of inclusions while reducing proteasome dysfunction in cell models of Huntington's disease (Bodner

et al., 2006). B2 has a similar effect on α-synuclein toxicity, suggesting a general protective role in protein misfolding diseases, such as Parkinson's disease. We report that B2 increases inclusion formation in cell models of SBMA. It is relevant to note that, unlike the case in Huntington's disease, B2 does not induce AR inclusion formation per se. In the absence of ligand, a condition in which there is no effect on inclusion formation, B2 increases the accumulation of mutant AR into microaggregates, further suggesting that inclusions and microaggregates behave differently. In contrast, B2 increases the deposition of mutant AR into inclusions in the presence of ligand. This suggests that the effect of B2 occurs at a stage that follows ligand binding. A unique feature of SBMA among the polyglutamine diseases is gender specificity. In SBMA, only males show full disease symptoms, and this is a result of high levels of circulating androgens in the serum. In the absence of ligand, AR is in the cytosol in an inactive state bound to Hsps, such as Hsp90, Hsp70, and Hsp40. Ligand binding induces a conformational change, which results in dissociation from the Hsps, translocation to the nucleus, and generation of inclusions. AR-positive inclusions have been found in motor neurons from SBMA patients (Li et al., 1998) as well as in cultured cells (Walcott and Merry, 2002). We explored the mechanism through which B2 increases formation of mutant AR-positive inclusions. We tested whether the induction of inclusion formation in the nucleus is a consequence of increased expression of the mutant protein or increased nuclear translocation. However, we did not find any difference in mutant AR expression or in the ligand-induced nuclear translocation in the presence or absence of B2. We also tested whether the mechanism through which B2 works involves the induction of the Hsps, such as Hsp90, Hsp70, and Hsp40. However, we could not detect any change in expression of these Hsps. From these results, we excluded the possibility that the effect of B2 on mutant AR toxicity and inclusion formation is due to an alteration of chaperone levels. These results are consistent with a previous report that B2 attenuates polyglutamine-expanded huntingtin toxicity through a mechanism that does not involve chaperone activity (Bodner et al., 2006).

Although inclusions had initially been considered toxic, the observations that accumulation of mutant huntingtin into inclusions in cultured striatal neurons inversely correlates with cell death (Arrasate et al., 2004) and that drugs that interfere with the ability of the cell to form inclusions cause cell death (Taylor et al., 2003) suggest a protective role for inclusions. Consistent with this model, mutant AR has been shown to accumulate more frequently and extensively in a diffuse nuclear pattern rather than in nuclear inclusions, with the extent of diffuse nuclear accumulation correlating with polyglutamine repeat length (Adachi et al., 2005). We show here that B2 decreases the toxicity of mutant AR not only in cultured cells but also in a fly model of SBMA. B2 had no effect on the toxicity induced by agents, such as staurosporin, that cause apoptosis independently of inclusion formation (Tamaoki et al., 1986; Matsumoto and Sasaki, 1989). However, we cannot exclude additional effects of B2 on cellular toxicity independent of polyglutamine inclusion formation. Our results provide the first evidence that B2 counteracts the toxicity of polyglutamine-expanded protein in vivo and suggest that agents that promote the deposition of unfolded proteins into inclusions may have therapeutic potential.

B2 Alters Mutant AR Function in Cultured Cells

Recent evidence suggests that altered protein function is an important component of polyglutamine disease pathogenesis (Lim et al., 2008). AR is a transcription factor activated by the male hormones testosterone and its derivative DHT (Poletti, 2004). Upon ligand binding, AR translocates to the nucleus to activate transcription of those genes whose regulatory regions contain specific androgen-responsive element sequences. Polyglutamine-expanded AR has been shown to have altered transcriptional activity in motor neuron-derived cells, which may contribute to disease pathogenesis (Lieberman et al., 2002). We found that B2 reduces AR transactivation in cultured cells without disrupting the general cellular transcription machinery. Although this is unlikely, B2 might have a repressive effect on the transactivation of other steroid receptors whose structure is similar to that of AR. Were this to occur, B21 might represent a valid alternative to B2. In fact, we found that B21 has no effect on normal AR transactivation, suggesting that it specifically targets the disease protein.

B2 inhibits activity of SIRT2 deacetylase, catalyzing the NAD^+-dependent reaction of acetyl group removal from lysine residues of protein substrates such as α-tubulin and histones (Outeiro et al., 2007). Acetylation of non-polyglutamine-expanded AR at the KXKK acetylation consensus site is important for AR transactivation (Fu et al., 2003) and is regulated by sirtuin activity (Fu et al., 2006). However, when we tested whether B2 affects transactivation of an acetylation-defective AR, we found that B2 is still active on this AR variant, indicating that B2 does not require this site to alter AR function. Rather, B2 may affect AR transcription by regulating acetylation of AR at different lysine residues or through a mechanism that is independent of AR acetylation. The observation that B2 increases the deposition of mutant AR into inclusions, while decreasing AR function and toxicity, leads us to speculate that, by increasing the compartmentalization of mutant AR into inclusions, B2 affects AR function and reduces mutant AR toxicity.

Is B2 a Potential Therapy for SBMA?

There is no effective therapy available for SBMA. A unique feature of SBMA among the polyglutamine diseases is gender specificity. This feature of SBMA has been reproduced in animal models of the disease, including mice (Katsuno et al., 2002; Yu et al., 2006) and flies (Takeyama et al., 2002; Pandey et al., 2007), and may

be attributed to androgen-dependent toxicity of the mutant AR protein. The androgen dependence of the disease offers the opportunity to develop therapy based on the reduction of testosterone levels in the serum. Indeed, reduction of testosterone levels by leuprorelin has had promising results in mouse models of SBMA (Katsuno et al., 2003) and, more recently, in a phase 2 clinical trial (Banno et al., 2009). However, the use of anti-androgens can be accompanied by several undesired side effects. We show here that B2 reduces the toxicity of mutant AR in cell cultures and fly models of SBMA. Based on these results, we propose B2 as a novel therapeutic approach for SBMA.

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