Discovery of a Novel, Monocationic, Small-Molecule Inhibitor of Scrapie Prion Accumulation in Cultured Sheep Microglia and Rov Cells

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Abstract

Prion diseases, including sheep scrapie, are neurodegenerative diseases with the fundamental pathogenesis involving conversion of normal cellular prion protein (PrP⁰) to disease-associated prion protein (PrPSc). Chemical inhibition of prion accumulation is widely investigated, often using rodent-adapted prion cell culture models. Using a PrPSc-specific ELISA we discovered a monocationic phenyl-furan-benzimidazole (DB772), which has previously demonstrated anti-pestiviral activity and represents a chemical category previously untested for anti-prion activity, that inhibited PrPSc accumulation and prion infectivity in primary sheep microglial cell cultures (PRNP 136VV/154RR/171QQ) and Rov9 cultures (VRQ-ovinized RK13 cells). We investigated potential mechanisms of this anti-prion activity by evaluating PrP expression with quantitative RT-PCR and PrP ELISA, comparing the concentration-dependent anti-prion and anti-pestiviral effects of DB772, and determining the selectivity index. Results demonstrate at least an approximate two-log inhibition of PrPSc accumulation in the two cell systems and confirmed that the inhibition of PrPSc accumulation correlates with inhibition of prion infectivity. PRNP transcripts and total PrP protein concentrations within cell lysates were not decreased; thus, decreased PrPSc expression is not the mechanism of PrPSc inhibition. PrPSc accumulation was multiple logs more resistant than pestivirus to DB772, suggesting that the anti-PrPSc activity was independent of anti-pestivirus activity. The anti-PrPSc selectivity index in cell culture was approximately 4.6 in microglia and 5.5 in Rov9 cells. The results describe a new chemical category that inhibits ovine PrPSc accumulation in primary sheep microglia and Rov9 cells, and can be used for future studies into the treatment and mechanism of prion diseases.

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Introduction

Prion diseases (transmissible spongiform encephalopathies [TSEs]) are progressive, fatal, transmissible, neurodegenerative diseases, which include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk, and various forms of Creutzfeld-Jakob disease (CJD) and kuru in humans [1]. The similarities between scrapie and CJD have long been recognized [2], and scrapie is the prototypical prion disease [3]; thus, scrapie is an experimental model that allows for the investigation of a natural prion disease in a natural host. The central feature of prion pathogenesis is the conversion of the normal cellular form of the host-encoded prion protein (PrP⁰) to an abnormal isoform, designated PrPSc (Sc superscript for sheep scrapie) [4,5,6]. This post-translational conversion involves a conformational change resulting in a detergent-insoluble, partially protease-resistant molecule that aggregates in affected cells and serves as the marker for prion diseases. PrPSc-accumulating cells include neurons and monocyte-derived cells (macrophages, microglia, and dendritic cells), among others [7,8,9,10,11].

Studies to identify anti-prion compounds often initially rely on inhibition of in vitro PrPSc formation [12]. Previous categories of compounds that have demonstrated anti-PrPSc activity in cell lines or animals include sulfated polyanions (e.g., pentosan polysulfate, dextran sulfate) [13,14,15,16,17,18], sulfonated dyes (e.g., congo red) [19,20,21,22], cyclic tetrapyroles (e.g., porphyrins) [23,24,25,26], polycyclic aromatic hydrocarbons (e.g., amphotericin B) [27,28,29,30,31], branched polyamines [32,33], quinolones and tricyclics (e.g., quinacrine) [12,34,35,36,37,38], polyenes (e.g., tetracycline) [39], and phosphorothioate oligonucleotides [40,41,42]. Currently, however, there are no effective treatments for prion diseases despite abundant investigation into therapeutics [43,44,45]. Continued investigation into new classes of anti-prion compounds is thus warranted, not only for the development of effective in vivo
Inhibition of Cultured Sheep Scrapie Prions

Materials and Methods

The Institutional Animal Care and Use Committee at Washington State University approved this study protocol (Permit numbers: #03811 and 03987). The ewe was euthanized by administering an intravenous overdose of sodium pentobarbital, in accordance with the 2007 American Veterinary Medical Association Guidelines on Euthanasia, and all efforts were made to minimize suffering.

Cells

Primary sheep microglial cells were obtained from a near-term Suffolk-cross fetus and cultured as previously described [49]. All cell media were made with pestivirus-free, fetal-bovine serum. Microglial cells were phenotyped via immunocytochemistry using the microglial markers biotinylated Ricinus communis agglutinin-1 (RCA-1) (Dako Cytomation) and an anti-CD14 antibody (MM61A, IgG1, VMRD, Inc.), as previously described [49]. A pellet of microglial cells was collected, washed by centrifugation, and used for genotyping the fetal prion gene as previously described [59].

Rov9 cells (B. Caughey with permission from D. Vilette) are rabbit renal epithelial cells (RK-13) stably transfected with the sheep VRQ (Val-136, Arg-154, Gln-171) allele of the prion gene under the control of a tetracycline-inducible promoter [46]. Rov9 cells were maintained in OMEM supplemented with 1 µg/ml doxycycline (OMEM-Doxy), as previously described [46]. PrPSc within Rov9Sc cells was verified by PrPSc-specific enzyme-linked immunosorbsent assay (ELISA) (see below).

Since Rov9 cells are derived from RK13 cells, Rov9 cells are permissive to BVDV infection [55]. Prior to inoculation microglial cells were confirmed BVDV negative and Rov9 cells were confirmed BVDV positive by RT-PCR and BVDV antigen ELISA (see below). The scrapie inoculum also contains infectious BVDV, and the preparation and application of PrPSc inoculum also transmits BVDV. Untreated microglial cells were used as controls for BVDV contamination.

Inoculation with PrPSc

For use as an inoculum, mechanical lysates of the Rov9Sc (Rov9 cells infected with PrPSc) and Rov9G (Rov9 cells not infected with PrPSc) cells were prepared as previously described [46]. Briefly, the Rov9 cells were grown to confluence in two 75-cm² plastic tissue culture flasks. Rov9Sc and Rov9G cells were rinsed three times with sterile 1× Dulbecco’s-PBS (D-PBS) and scraped into 10 ml of PBS. The cell pellets were collected by centrifugation at 2200 × g at room temperature for 7 min and resuspended in 0.5 ml of filter-sterilized 5% glucose. The cell suspensions were frozen and thawed four times and then subjected to 1 to 2 min of sonication in a cup horn sonicator. The inoculum was stored at −20°C. For inoculation, cells were passaged into twenty-well plates and allowed to grow to approximately 60% confluence. Cells were rinsed once with PBS and then overlaid with 200 µl of a 1/20 dilution of either the Rov9Sc lysate (microgliaSc) or the Rov9G lysate (microgliaG) in OMEM-MEM. MicrogliaSc (or Rov9Sc) and microgliaG (or Rov9G) were incubated for 6 hours, and then 200 µl of OMEM (or OMEM-Doxy) was added to each well. Following an additional 2 days of incubation, 0.5 ml of appropriate medium was added to each well, and cells were incubated for 4 days at which time they

Figure 1. Structure of 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772).
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were expanded into 25-cm² tissue culture flasks. Cells were fed every 3 or 4 days with appropriate medium as necessary and serially passaged 1/5 after reaching confluence.

DB772 inhibits PrPSc accumulation and prion infectivity

To eliminate BVDV from cultures 2-[2-benzimidazolyl]-3-[4-[2-imidazolino]phenyl]urane dihydrochloride (DB772) was used at 4 μM, as this is the concentration that cured primary bovine fibroblasts of pestivirus infection [56]. A 10 mM stock solution of DB772 was prepared in sterile water and stored at ~20°C until use. Rov9Sc, Rov9C, microgliaSc, and microgliaC were each split and cultured separately. There were 8 final treatment groups (Rov9Sc/DB772, Rov9Sc/UnTx, Rov9C/DB772, Rov9C/UnTx, microgliaSc/DB772, microgliaSc/UnTx, microgliaC/DB772, microgliaC/UnTx) [UnTx stands for untreated]. DB772 was maintained in the appropriate culture medium for four passages. Cells were then grown for an additional four passages without DB772 in the culture medium. Cells were collected after four passages with DB772 (P-4, “end of DB772 Tx”) and after four additional passages without DB772 (P-8, “end of clearance”) for prion infectivity quantification, PrPSc quantification, and PrPSc quantification (see below). Mechanical lysates of Rov9Sc/DB772 were also collected for prion inoculum creation (see above) to verify that prion infectivity was also inhibited by DB772.

DB772 concentration-dependence curve

To compare the concentration dependencies of BVDV and PrPSc, microgliaSc and Rov9Sc cells were treated with a dilution series of DB772. Samples were collected and analyzed for BVDV ELISA and PrPSc ELISA as described below. Cercosporin (Sigma-Aldrich, St. Louis, MO, USA), which does not inhibit PrPSc accumulation in Rov9Sc cells [47], was used as a negative control for non-specific cell death-induced PrPSc inhibition. Three independent experiments were analyzed. The 50% tissue culture effective concentration (TCEC50) was determined by nonlinear regression using a four-parameter logistic model for microgliaSc and a two-parameter exponential decay model for Rov9Sc (SigmaPlot ver. 11).

Sample collection

Cells in two 75-cm² flasks for each treatment group were trypsinized, resuspended, counted by light microscopy using a cytometer, and aliquoted appropriately for each assay. For ELISAs (BVDV, PrPSc), and total PrP, total protein evaluation, and RNA extraction, cells were collected by centrifugation at 2,300 g at room temperature for 5 min. The supernatant was aspirated and the cell pellet washed in D-PBS. Cells were collected again by centrifugation at 220 g at room temperature for 7 min, and the supernatant was aspirated.

For ELISAs and total protein measurements, cells were lysed in lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl [pH 8.0], 5 mM EDTA, and 150 mM NaCl) for 3 min at room temperature with gentle rocking, followed by centrifugation at 2,300 g at room temperature for 5 min. Cell lysates were stored at ~20°C until evaluation.

For RT-PCR, the washed cell pellet was lysed in buffer QLT (Qiagen, Valencia, CA, USA). Cell lysates were shredded (QiaShredder, Qiagen) and total RNA was purified using RNeasy mini spin columns (Qiagen) following manufacturer’s directions. Total RNA quantity and quality was determined by spectrophotometry (Thermo Scientific, ND-1000).

Total protein measurement

Since cells were collected based on a fraction of the cell suspension, total protein was analyzed to normalize samples for the various ELISAs. Aliquots of the cell lysates were diluted 1/5 into lysis buffer and then assayed for total protein using the bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific Inc, Rockford, IL, USA) following manufacturer’s directions for the microplate-based assay.

Pestivirus measurement

Pestivirus infection in cells was confirmed by RT-PCR and enzyme-linked immunosorbent assay (ELISA). To detect pestiviral RNA, 50 ng of total RNA was reverse transcribed into cDNA using random hexamers (SuperScript III First-Strand Synthesis System, Invitrogen, Carlsbad, CA, USA). The resulting cDNA was amplified using previous methods [60]. Briefly, DNA was amplified in a reaction consisting of 1 μl of cDNA template, 2 μM of each primer (Table 1), 12.5 μl of JumpStart™ RED-Taq® ReadyMix™ Reaction Mix (Sigma-Aldrich, St. Louis, MO, USA) and nuclease-free water to a final volume of 25 μl. The reaction was incubated in a thermocycler (C1000, Bio-Rad, Hercules, CA, USA) under the following conditions: 95°C/5 min; 35 cycles of 95°C/30 sec, 56°C/30sec, 72°C/30 sec; and 72°C/5 min. Isolation of detectable amounts of RNA was confirmed by amplifying glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA using intron-spanning primers (Table 1) [61] under the same PCR conditions described above. Amplification products were separated by agarose gel electrophoresis and visualized by staining with ethidium bromide. BVDV-free bovine turbinate cells were cultured in parallel with the Rov9 and microglial cells and served as negative controls.

BVDV antigen was detected by commercial ELISA (HerdChek®, BVDV Antigen Test Kit, IDEXX, Westbrook, ME, USA) following the manufacturer’s directions. Aliquots of cell lysates were diluted appropriately in lysis buffer to normalize protein concentrations (based on BCA results) and then added to the kit ELISA plate. The proprietary ELISA positive and negative controls were used per the manufacturer’s directions. Corrected optical density values were transformed to sample/positive ratio (S/P) using manufacturer’s directions: (sample-negative)/(positive-negative). A standard curve prepared from a half-log dilution series of BVDV-infected cell lysate was used to transform the S/P results to relative concentration of BVDV antigen. Untreated, prion-inoculated lysates at passage 4 were set at log 1, and all other results were normalized to this value. The relative amount of BVDV antigen within each passage was compared based on DB772 treatment status and PrPSc inoculation status using a two-way ANOVA followed by the Holm-Sidak method of multiple comparisons with a cutoff P value of 0.05 (SigmaPlot 11.2.0.5).

PrPSc ELISA and immunoblot assays

PrPSc was detected by commercial ELISA (HerdChek®, Scrapei Antigen Test Kit, IDEXX) following the manufacturer’s directions. Aliquots of cell lysates were diluted appropriately in lysis buffer to normalize protein concentrations (based on BCA results) and then added to the kit ELISA plate. The proprietary ELISA positive and negative controls were used per the manufacturer’s directions. A standard curve prepared from a half-log dilution series of Rov9Sc cell lysate was used to transform corrected optical density results to relative PrPSc concentrations. Untreated, prion-inoculated lysates at passage 4 were set at log 1, and all other results were normalized to this value. PrPSc concentrations within each passage were compared based on DB772 treatment status.
The specificity for samples derived from cultured sheep microglial cells and Rov9 cells has been previously confirmed by comparison with protease K-resistant immunoblotting [49]. In the present study, Rov9 cells were collected at passage five with and without 4 μM DB772 treatment (Rov9<sub>Sc/DB772</sub> and Rov9<sub>Sc/+DB772</sub>) for protease K treatment (50 μg/ml [2 units/ml]), phosphotungstic acid (PTA) precipitation, and immunoblotting (primary antibody P99/97.6.1), as previously described, [49] to again confirm that loss of ELISA signal was associated with loss of a protease K-resistant prion-specific protein band. Replicate aliquots of PTA-precipitated samples were electrophoresed and stained, following manufacturer’s directions, with SYPRO Ruby (Sigma-Aldrich) to confirm the precipitation of protein in the untreated and the DB772-treated samples.

**PRNP and PrP<sup>C</sup> measurement**

PRNP transcript levels were quantified using quantitative RT-PCR. RNA samples were treated with DNase (DNA-free kit, Ambion, Austin, TX, USA) followed by DNase Inactivation Reagent (Ambion) and centrifugation at 10,000×g for 1.5 min. Each treatment sample was tested in triplicate. One microgram of RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis Supermix for qRT-PCR (Invitrogen). Quantitative real-time PCR was performed in an iCycler iQ (Bio-Rad). The 20-μl reaction contained 1× SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), 200 nM of each specific primer (Table 1), 8 μl of 1/100 diluted cDNA (1 μg), and water. Reaction conditions were 50°C for 2 min, 95°C for 8.5 min, 40 cycles of denaturation at 95°C for 15 s and annealing at 59°C for 1 min followed immediately by a melt curve. A standard curve was used to transform C<sub>T</sub> values to relative concentrations and results were expressed as log<sub>2</sub> change (DB772-treated/untreated).

For PRNP transcripts and total PrP levels, the log<sub>2</sub> change was compared to the null hypothesis (no change) using individual one-sample t tests. A cutoff P value of 0.05 was used followed by the Bonferroni method for correcting for multiple comparisons (SigmaPlot).

**Cytotoxicity evaluation**

Cytotoxicity was measured in exponentially growing cell cultures. Two thousand cells were plated into a flat-bottomed 96-well plate for 24 h prior to addition of half-log dilutions of DB772. Cell viability, expressed as a percentage of untreated control, was determined after four days using 2-(4-iodonaphenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1) (Roche, United States), following manufacturer’s directions. The 50% cytotoxic (CC<sub>50</sub>) for microglia and Rov9 cells were determined from four independent experiments by nonlinear regression using a four-parameter logistic model (SigmaPlot). The percent cell viability at 4 μM, at the TCEC<sub>50</sub> (microglia: 2.4 μM; Rov9: 1.9 μM), and at 0 μM was compared within each passage, accounting for DB772-treatment status and PrP<sup>C</sup>-inoculation status, using a two-way ANOVA followed by the Holm-Sidak method of multiple comparisons with a cutoff P value of 0.05 (SigmaPlot).

**Results**

**DB772 effect on pestivirus**

To create BVDV-free cells, sheep microglial cells and ovinized rabbit epithelial cells (Rov9), both of which accumulate sheep-origin PrP<sup>C</sup>, were cultured in the presence of 4 μM DB772 for four passages. Continuous DB772 treatment of microglial cells (Fig. 2A; P-4) and Rov9 cells (Fig. 2B; P-4) for four passages significantly (P<0.001) reduced BVDV antigen to below detectable limits. Based on the relative minimum detection limits of the BVDV ELISA, as determined by the standard curves, the decrease in BVDV antigen is at least 0.3 logs in sheep microglial cells and 0.5 logs in Rov9 cells. Due to the relative insensitivity of the BVDV ELISA and the lack of detectable BVDV antigen, the absolute fold change may be higher. The negative BVDV ELISA results were validated by BVDV-specific RT-PCR, which failed to amplify a product from any of the DB772-treated groups (data not shown). Curing of BVDV from all replicates was not accomplished as after four additional passages in the absence of DB772 (P-8); BVDV antigen (Fig. 2) and nucleic acid (data not shown) returned to detectable levels in one DB772-treated microglial sample (Fig. 2A; P-8) a single microglial<sub>Sc/DB772</sub> sample and in all of the Rov9<sub>Sc/DB772</sub> and Rov9<sub>Sc/+DB772</sub> replicates (Fig. 2B; P-8). No influence of PrP<sup>C</sup> status on anti-pestivirus effectiveness was detected.

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**Table 1. RT-PCR primer information.**

| Target | Forward primer sequence | Reverse Primer Sequence | Amplicon size (bp) | Reference |
|-------|-------------------------|-------------------------|--------------------|-----------|
| BVDV<sup>a</sup> | ATGCCCT/ATAGTAGGACTAGCA | TCAAATCCATGTCGATGACTAC | 288     | [60] |
| GAPDH<sup>b</sup> | GAGGATGAGACCTTTGGCC | GTGAAAGCTGGAGATCAACG | 353     | [61] |
| PrP<sup>b</sup> | CCGTACCACCAACGATGT | CCGTACCACCAACGATGT | 159   | NA |
| GAPDH-Ov<sup>b</sup> | GGCCTGAAACTGGACTAGTAAA | CCCTCCAGATGCGAAAGT | 120     | [69] |
| GAPDH-Rab<sup>b</sup> | GCGCATACTGACCAGCAG | GAGTTCTCGGTTCGCTA | 147     | NA |

<sup>a</sup>RT-PCR primer set. <sup>b</sup>qRT-PCR primer set.

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Inhibition of Cultured Sheep Scrapie Prions

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DB772 effect on PrPSc

To determine if DB772 inhibits PrPSc accumulation, microgliaSc/DB772, microgliaSc/UnTx, Rov9Sc/DB772, and Rov9Sc/UnTx cells were assayed for PrPSc levels. The same time points that were used for BVDV detection were used for PrPSc detection. Treatment with 4 μM DB772 reduced PrPSc levels in cell lysates below detectable limits (Fig. 3A). Based on the relative minimum detection limit of the PrPSc ELISA, as determined by the standard curves, this decrease in PrPSc at P-4 is at least 1.8 logs for microgliaSc/DB772 cells and 2.2 logs for Rov9Sc/DB772 cells. After four clearance passages without DB772 (P-8) one microgliaSc/DB772 group and one Rov9Sc/DB772 group each contained minimal amounts of detectable PrPSc as determined by the standard curve (Fig. 3B).

The ELISA used in this study has been commercially validated for regulatory use and it has also previously been shown in sheep microglial cells and Rov9 cells that positive and negative ELISA results correspond appropriately with positive and negative proteinase K-resistant immunoblotting results [49]. We confirmed this conclusion specifically in this set of experiments by immunoblotting after DB772 treatment. At passage five, DB772-treated (4 μM) Rov9Sc/DB772 cell lysates lacked detectable proteinase K-resistant PrP, whereas the expected bands were detected in the Rov9Sc/UnTx cell lysate (Fig. 3C). As a control for the negative immunoblot signal, electrophoresed samples of PTA-precipitated lysates stained with SYPRO Ruby demonstrate the successful precipitation of proteins (Fig. 3D), confirming the specific loss of PrPSc in the DB772-treated samples.

DB772 effect on prion infectivity

A prion is ultimately defined by its infectious capability; thus, to confirm that the reduced PrPSc levels correlated with reduced prion infectivity, we compared prion infectivity between Rov9Sc/DB772 cultures and Rov9Sc/UnTx cultures (Fig. 4). Rov9 cells inoculated with the Rov9Sc/DB772-derived lysate failed to accumulate detectable PrPSc (0/9 were PrPSc positive); whereas, Rov9 cells inoculated with the Rov9Sc/UnTx-derived lysate consistently accumulated PrPSc (9/9 were PrPSc positive); thus, importantly demonstrating the loss of prion infectivity. Results are the mean of three independent treatments, each conducted in triplicate.

DB772 effect on PRNP transcript and PrP C expression levels

Since PrP C expression is required for PrPSc accumulation, one potential mechanism of DB772-mediated inhibition of PrPSc accumulation could be inhibition of PrP C expression. PRNP transcript and total prion protein (PrP) levels were assayed in DB772-treated cells to determine if they were decreased as compared to untreated cells. Levels of PRNP transcript (Fig. 5A) and total PrP (Fig. 5B) were not decreased in microglial cells or in microglia C/DB772 cells. In fact, at P-4 the PRNP transcript levels are significantly elevated in microgliaSc (P=0.003) and microgliaSc/DB772 (P<0.012), although increased total PrP expression could only be verified in microgliaSc/DB772 (P<0.001). Similarly, no decrease in PRNP transcript levels was detected in Rov9 cells. The trend towards an increase in PRNP transcript was also evident in Rov9 cells; however, the magnitude of change was less as compared to microglial cells, and only one group (Rov9Sc/DB772) showed statistical significance (Fig. 5C). Total PrP levels similarly failed to show a decrease upon DB772 treatment (Fig. 3D). In summary, expression of PrP C was not inhibited in DB772-treated microglial cells or Rov9 cells.

Concentration response of DB772 on pestivirus and PrPSc

To confirm the concentration dependence of the anti-PrPSc and anti-pestiviral effects, and to compare these concentration-dependent effects, microgliaSc and Rov9Sc cells were exposed to a range of DB772 concentrations, and the relative levels of PrPSc and pestivirus were determined. The anti-PrPSc and anti-pestiviral effects were concentration dependent. In initial experiments...
The dynamic range for anti-pestiviral activity was determined to be 0.004–0.04 μM in microgliaSc and 0.0004–0.004 μM in RovSc, whereas the dynamic range for PrPSc inhibition in microgliaSc and in RovSc was 0.4–4.0 μM (Table 2). Since the goal of this study was not to re-describe the anti-pestiviral effects of this compound [56], subsequent independent experiments focused solely on the anti-PrPSc concentration range. The final anti-PrPSc 50% tissue culture effective concentrations (TCEC50) for microgliaSc and for Rov9Sc was 2.4 ± 0.2 μM and 1.9 ± 0.4 μM, respectively (Table 2). This would represent a greater than two-log difference between the anti-PrPSc and anti-pestiviral TCEC50 values. In further independent experiments at higher concentrations, to describe the anti-PrPSc concentration dependence, complete anti-pestivirus activity was consistently measured at 0.4 μM (the lowest concentration used); thus, the anti-pestiviral TCEC50 must be lower than 0.4 μM and is different from the anti-PrPSc TCEC50 of 2.4 μM and 1.9 μM (microgliaSc and Rov9Sc, respectively) (Table 2).

Cytotoxicity evaluation

To determine the cytotoxicity of DB772 and the potential role of cell death as the mechanism of anti-prion action, microglial and Rov9 cells were exposed to half-log dilutions of DB772. The CC50 was calculated to be 10.6 ± 2.3 μM for microglial and 10.5 ± 1.4 μM for Rov9 cells (Table 2). The tissue culture selectivity index (CC50/EC50) for microglia and Rov9 cells is 4.6 and 5.5, respectively. The percent cytotoxicity was determined at relevant anti-prion concentrations: the anti-PrPSc TCEC50 (Microglia: 2.4 μM; Rov9: 1.9 μM), and at 4 μM (concentration used in Fig. 2). Since the planned ANOVA-based analysis failed the normality assumption, the Kruskal-Wallis one-way ANOVA based on ranks, with a Tukey method of multiple comparisons was
used. No change in cell viability was detected at the anti-PrPSc TCEC50 (Microglial % viability2.4μM: 98.2±2.1%; Rov9 % viability:1.9μM: 99.5±0.5%). At 4 μM (the concentration initially used to treat the cells) no cytotoxicity was definitively detected in microglial cells; however, early cytotoxic effects are likely at this concentration [Microglial % viability:4μM: 94.0±11%; P=0.079]. Significant cell death, however, was detected in Rov9 cells at 4 μM (Rov9 % viability: 4μM: 94.4±2.5%; P<0.001).

To further reduce the possibility of low levels of cytotoxicity being a non-specific cause of PrPSc inhibition, we tested the compound curcumin, which does not inhibit sheep scrapie in Rov9 cells [47]. Rov9 cells cultured in 100 and 56.4 μM of curcumin exhibited 100% cell death; thus, these samples could not be used to assay for PrPSc. The calculated curcumin CC50 was 68.6±20 μM in Rov9 cultures. Rov9 cells cultured at 31.7 μM subjectively exhibited significant cell death and the total protein levels in the 31.7 μM-treated samples (42.7±39.7 μg/ml) were significantly (P<0.001) lower than all sample groups treated with less curcumin (17.8 μM: 1032 μg/ml, 10 μM: 1116 μg/ml, 1 μM: 1105 μg/ml, and 0 μM: 1097 μg/ml). No anti-PrPSc TCEC50 can be calculated for curcumin as there was no inhibition of PrPSc accumulation, even at the clearly cytotoxic concentration of 31.7 μM. In fact, the relative levels of PrPSc in the treated groups were actually slightly higher than the untreated groups (Fig. 6).

Discussion

Despite previous research investigating compounds with anti-PrPSc activity [43,44,45], no effective chemotherapeutics exist for the treatment or prevention of prion diseases. Identification of new classes of anti-prion compounds is therefore vital, not only for the practical application of in vivo chemotherapeutics, but also for investigations studying the mechanisms of PrPSc conversion and accumulation. The data herein describe the discovery of in vitro anti-prion activity of a novel aromatic monocation. The anti-prion activity was demonstrated in two different cell culture models, including a cell type that is relevant to natural prion disease (sheep microglial cells). While the anti-prion effects described herein were discovered while using DB772 to eliminate BVDV from primary sheep microglial cells and Rov9 cells, to the authors’ knowledge there are no published reports of phenyl-turan-benzimidazoles cations with anti-prion activity.

The anti-PrPSc activity resulted in complete loss of PrPSc signal in sheep microglial cells and Rov9 cells by the end of the treatment (P-4), which paralleled a loss in prion infectivity. All replicates were not completely cured of PrPSc, however, as after four passages without DB772 (P-6), one group from each of the microglialSc/DB772 and Rov9Sc/DB772 samples had low levels of detectable PrPSc. Regardless, these results do demonstrate significant inhibition of sheep-derived PrPSc accumulation in two cell types.

In addition to the PrPSc inhibition, DB772 treatment also inhibited BVDV in both cell lines; however, it did not cure most of the cell replicates as BVDV antigen and BVDV RNA returned to detectable levels in one microglial replicate and in all of the Rov9 cell replicates. This incomplete pestivirus inhibition is different from what was demonstrated in primary bovine fibroblasts [56]. The differing results may be due to differences in the strains of BVDV that were tested, as well as the different cell types.

The cytotoxicity of DB772 was evaluated in sheep microglial cells and Rov9 cells. The 50% cytotoxicity point was similar between sheep microglial cells and Rov9 cells (10.5 μM and 10.0 μM, respectively) and is also similar to the previously demonstrated CC50 of 8.6 μM in B16 melanoma cells [58]. These values are in contrast to previous cytotoxicity studies using DB772 in Madin-Darby bovine kidney (MDBK) cells, in which the CC50 was substantially higher at 215 μM [57]. The discrepancy between these CC50 values is possibly a reflection of the different cell types, but may also be a result of the different culture conditions used.

Initial investigations into the mechanism of action were conducted and while no mechanism was identified, some potential mechanisms have been ruled out. Expression of PRNP is required for PrPSc permissiveness [62] and the level of expression correlates with PrPSc permissiveness [63,64]; thus, one obvious mechanism of PrPSc inhibition would be the partial to complete inhibition of PRNP expression. There was no evidence that DB772 inhibited PRNP expression, as PRNP transcript levels and total PrP protein levels were not decreased. In fact at passage four (the end of the DB772 treatment), microglialSc/DB772 and microglialC/DB772 cells have significant increases in PRNP transcript and total PrP protein levels as compared to the untreated controls. This confirms that DB772 does not inhibit PrPSc expression in microglial cells and suggests that PrPSc expression may increase in response to DB772 exposure. While the levels of PrPSc were increased, it is unclear if the increase is significant enough to be biologically relevant as the magnitude of change was small (less than two fold). Similarly, there was no evidence of PRNP expression inhibition in Rov9 cells as the direction of changes in PRNP transcript levels in Rov9 cells was towards an increase in PRNP transcript levels with DB772 treatment, and no change was identified in total PrP levels. The difference between the sheep microglial cells and Rov9 cells regarding PRNP transcript levels and total PrP levels is possibly attributed to the artificial PRNP expression system used in Rov9 cells (i.e., tetracycline-inducible promoter), which is unlikely to respond to the same stimuli as the natural PRNP promoter. This highlights the importance of using a natural prion cell culture model when investigating the mechanism of action of anti-PrPSc compounds.

Another potential mechanism considered for anti-PrPSc activity was the anti-pestivirus activity. To determine if the anti-PrPSc effects were related to the anti-pestiviral effects, the concentration dependencies of anti-PrPSc and anti-pestivirus were compared in Rov9 cells. Since the purpose of this paper was not to re-describe

Figure 4. DB772 inhibits prion infectivity in Rov9 cells. Rov9Sc/DB772 and Rov9Sc/UnTx cells were mechanically lysed and used as prion inoculum. Rov9 cells inoculated with these new inocula were then tested for PrPSc via ELISA. Data columns represent the corrected optical density (OD450 – OD620, per manufacturer’s instructions to correct for nonspecific optical density) means ± one standard deviation of three independent experiments, each run in triplicate. The y-axis reference line indicates the minimum detection limit of the ELISA. *, P<0.001. doi:10.1371/journal.pone.0051173.g004
the anti-pestirnal activity of DB772 [56,57], the anti-pestivirus TCEC50 and TCEC99 were not fully determined for these culture models. Instead, a single experiment was used to define the range of anti-pestivirus activity and follow-up experiments confirmed maximum anti-pestivirus activity at a concentration significantly different than the anti-PrPSc TCEC50. The effective anti-pestivirus

**Figure 5.** DB772 does not inhibit normal prion protein expression. Determination of DB772’s effect on PRNP transcript levels in primary sheep microglia (A) and Rov9 cells (C). RNA was collected at P-4 and P-8, and assayed via quantitative RT-PCR, using normalization to GAPDH. Columns represent the log2 change in DB772-treated groups compared to untreated groups from the same scrapie-treatment categories and time points (positive values indicate DB772 enhances PRNP transcript levels). Determination of DB772’s effect on total PrP concentration in primary sheep microglia (B) and Rov9 cells (D). Total protein was collected at P-4 and P-8, and assayed for total PrPC using a commercial ELISA. A standard curve was used to transform the corrected optical densities into relative concentration of PrPC. Columns represent the log2 change in DB772-treated groups compared to untreated groups from the same scrapie-treatment categories and time points. Results at each passage and for each scrapie status were statistically compared individually, using individual one-sample t tests, to the null hypothesis of no effect of DB772. *, P<0.0125.

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**Table 2.** Anti-PrPSc activity and cytotoxicity of DB772 in sheep microglial cells and Rov cells.

| Cells                  | BVDV TCEC50 (µM)       | PrPSc TCEC50 (µM)      | CC50 (µM)       |
|------------------------|------------------------|------------------------|-----------------|
| Sheep Microglia (DB772)| 0.004–0.04             | 2.4±0.2                | 10.6±2.3        |
| Rov9 (DB772)           | 0.0004–0.004           | 1.9±0.4                | 10.5±1.4        |
| Rov9 (curcumin)        | ND                     | NA                      | 68.6±20         |

*50% tissue culture effective concentration (TCEC50). Values are the range in one independent experiment; two additional independent experiments confirmed the value was less than 4 µM. *Values are the mean ± one standard deviation of three independent experiments. *50% cytotoxic concentration (CC50). Values are the mean ± one standard deviation of four independent experiments. *Not determined. *Not applicable as no anti-PrPSc activity was measured.

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Figure 6. Curcumin does not inhibit PrPSc accumulation, even at cytotoxic concentrations. Rov9Sc cells were exposed to a dilution series of curcumin for four days, and then an aliquot of cells was lysed for PrPSc ELISA. A standard curve was used to transform the corrected optical densities into relative concentration of PrPSc. Data points represent the means ± one standard deviation of four independent experiments. Rov9Sc/Con/ was set to 1, and all other points were normalized to this value. doi:10.1371/journal.pone.0051173.g006

ranges in microglia (0.004–0.04 μM) and Rov9 (0.0004–0.004 μM) are similar to the anti-pestivirous EC50 of 0.006±0.004 μM demonstrated in cultured bovine fibroblasts [56]. Replicate experiments, which focused on the anti-PrPSc range, confirmed that the anti-pestivirous TCEC50 (and thus TCEC99) is less than 0.4 μM. In contrast, the anti-PrPSc TCEC50 was approximately 2 μM in either cell type. Thus, the anti-PrPSc TCEC99 is at least five-fold higher than the anti-pestivirous TCEC99. Based on previous data [56] and our initial anti-pestiviral range results, however, the difference between the anti-PrPSc TCEC99 and the anti-pestivirous TCEC99 is more likely 50–500 fold. The data demonstrate that the anti-prion and anti-pestivirous effects clearly occurred at different concentration ranges; thus, it is unlikely that the inhibition of PrPSc accumulation is mediated by a loss of BVDV infection.

Non-specific anti-PrPSc activity associated with cell death was the final mechanism evaluated in this system. To test the effects of cytotoxicity on PrPSc accumulation, the selectivity index for DB772 was determined. Additionally, the cytotoxic and anti-PrPSc effects of curcumin, which does not inhibit PrPSc in Rov9 cells [47], were compared to DB772. DB772’s tissue culture selectivity index (SI) was 4.6 in microgliaSc and 5.5 in Rov9Sc cells. While the SI is low, it is similar to the SIs of many different anti-prion compounds including the often-studied quinacrine (SI: 4.5), amphotericin B (1.1), tannic acid (9.4), and cholesterol esterification modulators (1.5–11.4). It is, however, lower than others: dextran sulfate 500 (>250) and Congo red (>17) [63]. Furthermore, the SI is similar to other less commonly studied compound classes with potentially significant anti-prion activity such as diarylhiazoles [SI: 3.5] [66]. Our curcumin data indicate that cell death does not necessarily dictate a decrease in PrPSc levels and that non-specific effects associated with cell death do not cause the anti-PrPSc effects in this study, i.e., while the anti-prion mechanism of DB772 may or may not be closely related to its toxicity, there is no evidence that cell death is the mechanism of the inhibition. Future studies into analogues of DB772, of which there are many already available [56,57], may identify related compounds with larger anti-PrPSc selectivity indices.

While the anti-PrPSc mechanism of action is not determined in this study, we have excluded several possible mechanisms. One potential way to investigate the anti-PrPSc activity is to extrapolate from the anti-pestiviral agent mechanisms of related compounds. As an example, furamidine, [(2,5-bis-(4-amidophenyl)-furian], a molecule related to DB772, demonstrates resistance against pestiviral parasites including Plasmodium sp. and Trypanosoma sp. [58]. The anti-pestiviral [58] activity of the analogues of DB772, including furamidine, is thought to be mediated by DNA binding. What role nucleic acid binding may have in the DB772-mediated inhibition of PrPSc accumulation is unclear; however, it could be postulated that the DNA-binding capability results in altered transcription of genes [67], which then impacts PrPSc accumulation. Regarding DB772, its nuclear uptake is impaired relative to many other furamidine analogues [58] but little else is known about its activity. It is thus difficult at this time to speculate about any intracellular mechanism of anti-PrPSc activity of DB772.

Fortunately, due to the anti-pestiviral potential of these compounds, a library of related compounds already exists. Additionally, due to the anti-pestiviral activity, in vivo work on the anti-pestiviral efficacy and pharmacokinetics has been initiated in cattle [68]. Structure-activity relationship studies are ongoing with the aims of identifying more selective anti-prion molecules, elucidating the mechanisms of action, and determining if the anti-prion activity is therapeutically or mechanistically relevant.

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Author Contributions

Conceived and designed the experiments: JBS DAS TVB DWB. Performed the experiments: JBS KDD BFB BAM AK. Analyzed the data: JBS DAS KDD BFB. Contributed reagents/materials/analysis tools: DWB AK. Wrote the paper: JBS DAS KDD TVB BAM DWB.

References

1. Lasmezas CI (2003) The transmissible spongiform encephalopathies. Rev Sci Tech 22: 23–36.
2. Klatoi I, Gadjuseck DC, Zigaš V (1959) Pathology of Kuru. Lab Invest 8: 799–847.
3. Prusiner SB (1982) Novel proteinaceous infectious particles cause scrapie. Science 216: 136–144.
4. Borchart D, Scott M, Taraboulos A, Stahl N, Prusiner SB (1990) Scrapie and cellular prion proteins differ in their kinetics of synthesis and topology in cultured cells. J Cell Biol 110: 743–752.
5. Cauphey B, Raymond GJ (1991) The scrapie-associated form of PrP is made from a cell surface precursor that is both protease- and phospholipase-sensitive. J Biol Chem 266: 18217–18223.
6. Basler K, Oesch B, Scott M, Westaway D, Walchli M, et al. (1988) Scrapie and cellular PrP isoforms are encoded by the same chromosomal gene. Cell 46: 417–420.
7. Gilch S, Schmitz F, Aguilh Y, Kehler C, Bulow S, et al. (2007) Cpg and LPS can interfere negatively with prion clearance in macrophage and microglial cells. FEMS J 274: 5834–5841.
8. Prinz M, Montrasio F, Klein MA, Schwarz P, Priller J, et al. (2002) Lymph nodal prion replication and neuroinvasion in mice devoid of follicular dendritic cells. Proc Natl Acad Sci U S A 99: 919–924.
9. Huang FP, Farquhar CF, Mabbott NA, Bruce ME, MacPherson GG (2002) Migrating intestinal dendritic cells transport PrPSc from the gut. J Gen Virol 83: 267–271.
10. Baker CA, Martin D, Mandelis L (2002) Microglia from Creutzfeldt-Jakob disease-infected brains are infectious and show specific mRNA activation profiles. J Virol 76: 10905–10913.

11. Iwamaru Y, Takenouchi T, Oghara K, Hoshino M, Takata M, et al. (2007) Synergistic effects of dexamethasone and ribavirin on porcine pestiviruses. J Virol 81: 4842–4850.

12. Vercellotti G, Raffi L, Novelli C, Rizzetto M, Boldrini M, et al. (2001) Alpha(1) antitrypsin binds differentially to prion proteins and affects their intracellular metabolic fate. J Biol Chem 276: 30758–30765.

13. Supattapone S, Wille H, Uyechi L, Safar J, Tremblay P, et al. (2001) Branched phthalocyanine tetrasulfonate in scrapie-infected mice. J Infect Dis 188: 699–702.

14. Kimberlin RH, Walker CA (1986) Suppression of scrapie infection in mice by an amphotericin B analogue, delays the appearance of spongiosis, astrogliosis and PrPres accumulation in the brain of scrapie-infected hamsters. J Comp Pathol 121: 1133–1139.

15. Ladogana A, Casaccia P, Ingrosso L, Cibati M, Salvatore M, et al. (1992) Heparin-like molecules bind differentially to prion proteins and change their intracellular metabolic fate. J Cell Physiol 157: 319–325.

16. Vercellotti G, Raffi L, Novelli C, Rizzetto M, Boldrini M, et al. (2001) Alpha(1) antitrypsin binds differentially to prion proteins and affects their intracellular metabolic fate. J Biol Chem 276: 30758–30765.

17. Kocisko DA, Caughey WS, Race RE, Roper G, Caughey B, et al. (2006) A strategy to test for the efficacy of potential therapeutic agents against scrapie. J Gen Virol 87: 39–50.

18. Priola SA, Raines A, Caughey W (2003) Prophylactic and therapeutic effects of cycloheximide on scrapie. J Gen Virol 84: 2307–2316.

19. Caughey B, Raymond LD, Horiuchi M, Caughey B (1998) Inhibition of scrapie by impairment of agent replication in spleen. J Gen Virol 79: 1821–1829.

20. Rudyk H, Vasiljevic S, Hennion RM, Birkett CR, Hope J, et al. (2000) Screening Congo Red and its analogues for their ability to prevent the formation of PrP(Sc) in scrapie-infected cells. J Gen Virol 81: 1155–1164.

21. Demaimay R, Adjou K, Lasmezas C, Lazarini F, Cherifi K, et al. (1994) Congo red prolongs the incubation period in scrapie-infected hamsters. J Virol 69: 506–508.

22. Caughey B, Raymond LD, Horiuchi M, Caughey B (1998) Inhibition of scrapie-resistant prion protein formation by porphyrins and phthalocyanines. Proc Natl Acad Sci U S A 95: 12117–12122.

23. Caughey WS, Raymond LD, Horiuchi M, Caughey B (1998) Inhibition of scrapie-resistant prion protein formation by porphyrins and phthalocyanines. Proc Natl Acad Sci U S A 95: 12117–12122.

24. Priola SA, Raines A, Caughey W (2003) Prophylactic and therapeutic effects of phthalocyanine tetrasulfonate in scrapie-infected mice. J Infect Dis 188: 699–702.

25. Priola SA, Raines A, Caughey W (2003) Prophylactic and therapeutic effects of phthalocyanine tetrasulfonate in scrapie-infected mice. J Infect Dis 188: 699–702.

26. Kocisko DA, Baron GS, Rubinstein R, Chen J, Kuizon S, et al. (2003) New Inhibitors of Scrapie-Associated Prion Protein Formation in a Library of 2,000 Drugs and Natural Products. J Virol 77: 10289–10294.

27. Doh-Ura K, Iwaki T, Caughey B (2000) Lysosomotropic agents and cysteine proteinase inhibitors inhibit scrapie-associated prion protein accumulation. J Virol 74: 4849–4857.

28. Kocisko DA, Caughey B (2006) Mefloquine, an antimalaria drug with antiprion activity in vitro, lacks activity in vivo. Journal of virology 80: 1044–1046.

29. Barres A, Tagliafico F, Feletti G, Blaze C, Salomone M, et al. (2003) Evasion of quinacrine treatment for prion diseases. J Virol 77: 8462–8469.

30. Kocisko DA, Caughey B (2006) Mefloquine, an antimalaria drug with antiprion activity in vitro, lacks activity in vivo. Journal of virology 80: 1044–1046.

31. Murakami-Kubo I, Doh-Ura K, Ishikawa K, Kawatake S, Sasaki K, et al. (2004) Quinoline derivatives are therapeutic candidates for transmissible spongiform encephalopathies. J Med Chem 47: 3181–3188.

32. Ghaemmaghami S, May BC, Rendo AR, Prusiner SB (2010) Discovery of 23 aminothiazoles as potent antiprion compounds. Journal of virology 84: 3408–3412.

33. Supattapone S, Wille H, Uyechi L, Safar J, Tremblay P, et al. (2001) Branched phthalocyanine tetrasulfonate in scrapie-infected mice. J Infect Dis 188: 699–702.

34. Priola SA, Raines A, Caughey W (2003) Prophylactic and therapeutic effects of phthalocyanine tetrasulfonate in scrapie-infected mice. J Infect Dis 188: 699–702.

35. Doh-Ura K, Iwaki T, Caughey B (2000) Lysosomotropic agents and cysteine proteinase inhibitors inhibit scrapie-associated prion protein accumulation. J Virol 74: 4849–4857.

36. Kocisko DA, Caughey B (2006) Mefloquine, an antimalaria drug with antiprion activity in vitro, lacks activity in vivo. Journal of virology 80: 1044–1046.

37. Murakami-Kubo I, Doh-Ura K, Ishikawa K, Kawatake S, Sasaki K, et al. (2004) Quinoline derivatives are therapeutic candidates for transmissible spongiform encephalopathies. J Med Chem 47: 3181–3188.

38. Ghaemmaghami S, May BC, Rendo AR, Prusiner SB (2010) Discovery of 23 aminothiazoles as potent antiprion compounds. Journal of virology 84: 3408–3412.

39. Kocisko DA, Vaillant A, Lee KS, Arnold KM, Berholet N, et al. (2006) Potent antiprion activities of degenerate phosphorothioate oligodeoxynucleotides. Antimicrobial agents and chemotherapy 50: 1034–1044.

40. Sethi S, Lipford G, Wagner H, Kretzschmar H (2002) Postexposure prophylaxis against prion disease with a stimulator of innate immunity. Lancet 360: 229–230.

41. Setti S, Lipford G, Wagner H, Kretzschmar H (2002) Postexposure prophylaxis against prion disease with a stimulator of innate immunity. Lancet 360: 229–230.

42. Karup MV, Giles K, Gelhart-Niv S, Scott MR, Linuppova VR, et al. (2007) Phosphorothioate oligodeoxynucleotides reduce PrP levels and prion infectivity in vitro. J Virol 81: 190–192.

43. Caughey B, Caughey WS, Kocisko DA, Lee KS, Silveira JR, et al. (2006) Prions and transmissible spongiform encephalopathy (TSE) chemotherapeutics: A common mechanism for anti-TSE compounds? Accounts of chemical research 39: 234–242.

44. Trevitt CR, Collinge J (2006) A systematic review of prion therapeutics in experimental models. Brain: a journal of neurology 129: 2241–2253.

45. Dormont D (2003) Approaches to prophylaxis and therapy. British medical bulletin 66: 201–292.

46. Vinkovic D, Andreoletti O, Archer F, Madelaine MF, Vilotte JL, et al. (2001) Ex vivo propagation of infectious sheep scrapie agent in heterologous epithelial cells expressing ovine prion protein. Proc Natl Acad Sci U S A 98: 4055–4059.

47. Kocisko DA, Engel AL, Harbuck K, Arnold KM, Olsen EA, et al. (2005) Comparison of prophylactic treatments for scrapie with two strains of mouse and sheep scrapie. Neuroscience letters 388: 106–111.

48. Ladogana A, Casaccia P, Ingrosso L, Cibati M, Salvatore M, et al. (1992) Heparin-like molecules bind differentially to prion proteins and change their intracellular metabolic fate. J Cell Physiol 157: 319–325.
68. Newcomer BW, Marley MS, Galik PK, Walz PH, Zhang Y, et al. (2012) Antiviral treatment of calves persistently infected with bovine viral diarrhea virus. Antiviral chemistry & chemotherapy 22: 171–179.

69. Budhia S, Haring LF, McConnell I, Blacklaws BA (2006) Quantitation of ovine cytokine mRNA by real-time RT-PCR. J Immunol Methods 309: 160–172.