A simple in vitro assay for assessing the efficacy, mechanisms and kinetics of anti-prion fibril compounds

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ABSTRACT

Prion diseases are caused by the conversion of normal cellular prion proteins (PrP) into lethal prion aggregates. These prion aggregates are composed of proteinase K (PK) resistant fibrils and comparatively PK-sensitive oligomers. Currently there are no anti-prion pharmaceuticals available to treat or prevent prion disease. Methods of discovering anti-prion molecules rely primarily on relatively complex cell-based, tissue slice or animal-model assays that measure the effects of small molecules on the formation of PK-resistant prion fibrils. These assays are difficult to perform and do not detect the compounds that directly inhibit oligomer formation or alter prion conversion kinetics. We have developed a simple cell-free method to characterize the impact of anti-prion fibril compounds on both the oligomer and fibril formation. In particular, this assay uses shaking-induced conversion (ShIC) of recombinant PrP in a 96-well format and resolution enhanced native acidic gel electrophoresis (RENAGE) to generate, assess and detect PrP fibrils in a high throughput fashion. The end-point PrP fibrils from this assay can be further characterized by PK analysis and negative stain transmission electron microscopy (TEM). This cell-free, gel-based assay generates metrics to assess anti-prion fibril efficacy and kinetics. To demonstrate its utility, we characterized the action of seven well-known anti-prion molecules: Congo red, curcumin, GN8, quinacrine, chloropromazine, tetracycline, and TUDCA (taurourspdeoxycholic acid), as well as four suspected anti-prion compounds: trans-resveratrol, rosmarinic acid, myricetin and ferulic acid. These findings suggest that this in vitro assay could be useful in identifying and comprehensively assessing novel anti-prion fibril compounds.

Abbreviations: PrP, prion protein; PK, proteinase K; ShIC, shaking-induced conversion; RENAGE, resolution enhanced native acidic gel electrophoresis; TEM, transmission electron microscopy; TUDCA, tauroursdeoxycholic acid; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; CJD, Creutzfeldt Jakob disease; GSS, Gerstmann–Sträussler–Scheinker syndrome; FFI, fatal familial insomni; recPrP, recombinant monomeric prion protein; PrPSc, infectious particle of misfolded prion protein; RT-QuIC, real-time quaking-induced conversion; PMCA, Protein Misfolding Cyclic Amplification; LPS, lipopolysaccharide; EGCG, epigallocatechin gallate; GN8, 2-pyrrolidin-1-yl-N-[4-[2-pyrrolidin-1-yl-acetylamino]-benzyl]-phenyl]-acetamide; DMSO, dimethyl sulfoxide; ScN2A, scrapie infected neuroblastoma cells; IC50, inhibitory concentration for 50% reduction; recMoPrP23-231, recombinant full-length mouse prion protein residues 23-231; EDTA; PICUP, photo-induced cross-linking of unmodified protein; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride.

Introduction

Prion diseases are transmissible spongiform encephalopathies that include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, as well as Kuru, Creutzfeldt Jacob Disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS) and Fatal Familial Insomnia (FFI) in humans [1]. All of these diseases are caused by misfolding of a non-infectious, endogenous, helix-rich prion protein (PrP(C)) into an infectious β-sheet rich prion form (PrP(Sc)). PrP(C) is a highly conserved membrane-bound protein that is widely expressed in most tissues, but enriched in neuronal synaptic membranes [2]. Despite many large-scale anti-prion drug screening efforts to identify new candidate molecules, there are currently no pharmaceuticals available to treat prion diseases [3–8]. Indeed, no significant improvement in
patient outcomes has been found in human clinical trials testing such promising anti-prion compounds as flupirtine [9], pentoxyfiban [10], quinacrine [11,12] and doxycycline [13–15].

Bioassays to identify effective anti-prion molecules have typically been performed using mouse models of prion disease, scrapie infected organotypic brain slices or chronically infected cell culture [5,16–18]. All three types of assays are designed to measure endpoints (survival for mouse assays, PrPSc content for the other two assays) rather than mechanisms. Further, all three assays require strict biohazard containment, significant resources, highly specialized facilities and can take weeks (cell culture or organotypic brain slices) or months to complete. Mouse bioassays are thought to be the closest preclinical test we have for determining if an anti-prion molecule is an effective prion disease therapy or prophylactic. However most anti-prion molecules that are effective for mouse scrapie strains (eg. Rocky Mountain Laboratory (RML) strain) are not effective for human prions (eg. GSS isolates) [19,20]. Bioassays using organotypic brain slices are particularly appealing because they avoid the challenges of working with live animal models and they mimic the neuronal cell architecture needed for prion propagation more closely than cell culture. However, organotypic brain slices require special microsurgical and tissue handling skills as well as containment facilities that are not easily found or readily available. As a result, cell-based assays, because of their relative simplicity, are the most common anti-prion screening tool. Cell-based methods measure which anti-prion molecules reduce the level of PrPSc via Western blotting. In particular, these assays typically use anti-prion antibodies to detect PrPSc from proteinase K (PK) digested brain homogenate [3,5,21]. Unfortunately, even these ‘easier’ cell-based assays require specialized cell culturing facilities, extensive training and appropriate containment.

All of the above-mentioned anti-prion assays are end-point assays only. They do not measure mechanisms or provide much information regarding the molecular details of prion misfolding. Therefore, it would also be desirable to have an assay that measures not only end-points, but also mechanisms. This is because the mechanism of action for experimental anti-prion drugs has a significant impact on their route of delivery, formulation and ultimately their success. One class of assays that offers information about the mechanism of action of anti-prion compounds is the cell-free prion conversion and propagation assay. Unlike anti-prion screening assays, which measure end-points using living cells or tissues, cell-free prion conversion and propagation assays indicate if a drug directly acts on PrP rather than through other cellular factors. Propagation assays typically use small amounts of animal-derived infectious prions (PrPSc) that are added to large amounts of native (often recombinant) PrPC and the mixture is shaken or sonicated for several days. Common prion propagation techniques include protein misfolding and cyclic amplification (PMCA) and real-time quaking-induced conversion (RT-QuIC). Conversion and propagation are normally measured by the PK-resistance of the resulting PrPSc fibrils or via Thioflavin T fluorescence. Unfortunately, most cell-free prion conversion and propagation assays use detergents and other denaturants [22,23] and these denaturants frequently block or disrupt the interaction of ligands with PrP targets [24]. This makes these cell-free methods inappropriate for performing anti-prion screening assays.

As noted, all of the above-mentioned assays have some shortcomings. The animal, tissue and cell-culture assays for anti-prion compound screening require long periods of time, specialized training, expensive or difficult-to-find equipment and biohazard containment. On the other hand, the cell-free, propagation assays, while being easier to perform and providing some mechanistic information, are not amenable for anti-prion screening because of their use of detergents. Ideally, what is needed is a simplified anti-prion compound-screening assay that uses no detergents, mimics physiological conditions and requires little time (hours), few resources, modest training and minimal containment. We believe we have developed such an assay.

Previously we described a shaking-induced conversion (ShIC) method to convert recombinant PrPSc de novo, to synthetic β-sheet rich oligomers and fibrils, without detergents, under near physiological conditions [25]. These synthetic PrP fibrils lack the pathologically infectious nature of prion (PrPSc) particles, but share the increase in β-sheet structure and PK resistance seen in prions. This method does not require the addition of infectious prion seeds, as is the case for QuIC and PMCA. Separately, we also described a method to monitor the time-dependent formation of PrP oligomers and fibrils with resolution enhanced native acidic gel electrophoresis (RENAGE) [25,26]. The near physiological conditions in ShIC allow one to monitor native interactions of anti-prion compounds with PrP isoforms. Given the potential of ShIC to permit the rapid, in vitro assessment of anti-prion compounds under physiological conditions and given the potential of RENAGE to monitor the mechanisms of action and PrP fibril propagation kinetics, we hypothesized that by combining the two techniques it would be possible to develop a simplified anti-prion, and more specifically
anti-prion fibril assay that requires little time, few resources, modest training, minimal containment and which measures not only end-points, but also mechanisms. The designation as an anti-prion fibril assay is used to distinguish these compounds as modulating synthetic PrP oligomer and fibril formation in comparison to anti-prion compounds that inhibit formation of infectious prion particles.

To shorten the time requirement, the ShIC method that we previously described [25] was optimized so that the time to convert monomer to oligomers and fibrils was reduced to 24 hours and the assay could be performed in a 96-well format. We then optimized the RENAGE technique so that the gel analysis could be performed more consistently and multiple gels ran at the same time. We found that this simple and easy-to-perform cell-free assay generates not only a measure of the efficacy of the anti-prion fibril compounds that matches the results of standard in vivo anti-prion assays, but it also provides four useful metrics to assess other anti-prion fibril activities. In particular this new assay is able to measure: 1) small oligomer abundance; 2) large oligomer abundance; 3) fibril growth lag phase and 4) fibril growth rate. To confirm the results seen through this simple cell-free assay, further characterization by PK analysis and negative stain transmission electron microscopy (TEM) was also performed. A much more detailed description of the methods and the results follows.

Results

The combination of ShIC and RENAGE provides a unique system for not only detecting (or confirming) promising anti-prion fibril compounds but also for uncoupling the kinetic and mechanistic details of how anti-prion compounds act on prions during their conversion and propagation. During prion conversion and propagation, it is known that monomeric PrPC forms a distribution of misfolded PrP aggregates [27]. Using RENAGE it is possible to rapidly separate and quantify PrP monomers, oligomers and fibrils [26]. Previously we showed that RENAGE can be used to analyze cell-free recombinant PrP conversion using urea, acidic pH, lipopolysaccharide (LPS) or detoxified LPS as the catalysts for prion conversion [25,26,28,29]. We also showed that RENAGE can be used detect or quantify the formation of β-sheet rich PrP oligomers and/or fibrils. In this study we sought to develop a method to effectively determine if anti-prion molecules, identified in cell-based bioassays, disrupt or modify PrP oligomer and fibril formation. To test the performance of this method we chose a collection of small molecules that are known anti-prion compounds as well as several unknown molecules, some of which were expected to exhibit anti-prion activity (Figure 1). The compounds that we selected were Congo red, curcumin, GN8, quinacrine, chlorpromazine, tetracycline, TUDCA (tauroursodeoxycholic acid), trans-resveratrol, rosmarinic acid, myricetin, ferulic acid, gallic acid and melatonin.

Identification of an optimal cell-free conversion protocol for screening anti-prion fibril compounds

Our previous work had shown that cell-free de novo prion conversion could be performed with recombinant PrPC using four different ‘catalyzing’ conditions including: 1) a urea mixture (3 M urea, 20 mM sodium acetate pH 4 with 200 mM NaCl), 2) an acidic shock treatment (pH 1 in water), 3) a solution containing LPS (1:1 weight ratio of PrP to LPS) and 4) shaking-induced conversion or ShIC (shaking at 250 rpm in 20 mM sodium acetate pH 5.5 for 48 hours). To identify which cell-free conversion/propagation protocol was best for detecting or screening anti-prion compounds we tested each of the conversion/propagation processes using several well-known anti-prion compounds (Congo red, curcumin, tetracycline and quinacrine) [30–36]. We used RENAGE to assess which conversion protocols were modulated by the known anti-prion compounds as reported in cell or animal models. We found that shaking-induced conversion (ShIC) very closely mirrored the expected results of these anti-prion compounds. As seen in Figure 2, RENAGE of Syrian hamster PrP80–232 (a recombinant prion construct consisting of residues 90–232) converted by ShIC in the presence of these anti-prion compounds showed the expected strong anti-prion effects for all the tested compounds (increasing or decreasing the formation of oligomers and fibrils). In contrast, with urea-induced conversion, only curcumin exhibited the expected anti-prion effects [26] (Suppl. Figure 1). With acid-induced or acid-shock conversion, only modest anti-prion effects were seen with curcumin and tetracycline (Suppl. Figure 1). Furthermore, the variability in the distribution of oligomers formed by the acid-shock treatment made it difficult to properly assess the effects of different anti-prion drugs. Almost no effects on LPS-induced conversion of PrP80–232 were observed using most of the anti-prion molecules. However, epigallocatechin gallate (EGCG) and myricetin incubated with PrP80–232 caused the formation of large oligomers near the top of the separating gel in the presence and absence of LPS (Suppl. Figure 2). Interestingly, EGCG caused visible
precipitation with the LPS-conversion assay. Overall, we found that ShIC provides the most robust system to measure and assess anti-prion-induced changes in PrP oligomer and fibril formation. Furthermore, ShIC is a detergent or additive-free assay, meaning that it more closely resembles physiological conditions.

**Optimized conditions for PrP ShIC in a 96-well assay**

The ShIC conditions were then optimized to allow efficient conversion of full-length recMoPrP$^{23-231}$ (a recombinant mouse prion construct consisting of residues 23-231) within a 24 to 36 hour period, in a

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**Figure 1.** Chemical structures of small molecules tested for anti-prion activity: (a) Congo red, (b) curcumin, (c) GN8 (2-pyrrolidin-1-yl-N-[4-[4-(2-pyrrolidin-1-yl-acetylamino)-benzyl]-phenyl]-acetamide), (d) quinacrine, (e) chlorpromazine, (f) TUDCA (tauroursodeoxycholic acid), (g) tetracycline, (h) rosmarinic acid, (i) resveratrol, (j) myricetin, and (k) ferulic acid.
We found that ShIC occurs efficiently in Nunc Deep well polypropylene plates sealed with a Well Cap and shaken with the plane of the plate placed at 90° (vertical). However, aggregation did not occur when these 96-well plates were shaken horizontally (along the plane of the plate). Furthermore, ShIC did not occur in Nunc polypropylene conical plates or Costar polystyrene flat bottom plates, either in vertical or horizontal format. We also determined that ShIC of recMoPrP \(^{23-231}\) occurs at 0.2, 0.3, 0.4 and 0.5 mg/mL in a 1.5 mL tube, with the conversion at any concentration above 0.2 mg/mL going to completion after 20 hrs. Therefore, to reduce the amount of purified recMoPrP \(^{23-231}\) required we chose to use 0.3 mg/mL recMorPrP for the 96-well ShIC assay.

Because many anti-prion drug candidates are not highly soluble in water and many standard drug formulations include other organic solvents, we also tested if solvents used for solubilizing anti-prion drugs would affect the kinetics of oligomer formation and fibril growth in our ShIC assay. We tested dimethyl sulfoxide (DMSO) and methanol. Comparison of ShIC with 0.77% methanol or 0.77% DMSO (vol:vol) by RENAGE showed a similar degree of oligomer and fibril formation. Further kinetic analysis of ShIC at 0.5 mg/mL recMoPrP \(^{23-231}\) with 1.9% methanol or 1.9% DMSO showed that conversion in the presence of methanol was most similar to the control (result not shown). As a result, we decided that all drugs would be dissolved in methanol such that the final methanol concentration for the ShIC assays would be between 0.26% to 0.92% (vol:vol). This would correspond to recMoPrP \(^{23-231}\) to drug molar ratios of 1:1 (0.26% methanol), 1:2 (0.46% methanol), 1:4 (0.46% methanol) or 1:8 (0.92% methanol).

**Time course measurement of PrP monomer, oligomer and Fibril abundance**

The time course for the changes in PrP monomer, oligomer and fibril populations during ShIC, in the presence of anti-prion molecules, was monitored by RENAGE. Data for multiple time points were required because the kinetic attributes of fibril formation, whether by templated misfolding or nucleation dependent polymerization, restrict the ability to compare the influence of drugs at a single time point. Figure 3(a) shows a RENAGE gel collected for a sample of 0.3 mg/mL recMoPrP \(^{23-231}\) in 20 mM sodium acetate pH 5.5 and with 0.46% methanol, that was shaken at 280 rpm for 0, 2, 5, 8, 11, 12, 15, 18, 21, 24, 30 and 36 hours. At time zero, the recMoPrP \(^{23-231}\) sample is purely monomeric and then after shaking it is converted to a collection of oligomers (bands above the monomer) and then later to a fibril band at the top of the stacking gel. Previous studies have shown that the fibril band seen in RENAGE gels corresponds to PrP fibrils as visualized by negative-stain TEM [25]. The quantity of monomers, small oligomers, large oligomers and fibrils were measured by densiometric analysis, where each gel lane was
converted to a chromatogram and the ratios of the peak areas were determined computationally using Origin software. Peak areas were normalized based on the peak intensity of a 2 µg recMoPrP$^{23-231}$ monomer band and standardized across different gels using the peak area of the truncated recMoPrP$^{90-231}$ large oligomer standard (Figure 3(a), lane 1). Oligomeric peaks were grouped into two categories: 1) small oligomer bands between 8-mers to 12-mers and 2) large oligomers greater than 16-mers. The peak areas are plotted versus time for these four PrP isoforms (Figure 3(b)). The decrease in the PrP monomer population and the increase in the PrP fibril isoform were fit to sigmoidal functions (Equation 1), where x corresponds to time, a is the maximum amplitude, and xc is the time value at half. The time-dependency of small oligomer and large oligomer populations were fit with a b-spline function.

$$y = a/(1 + \exp(-k^c(x-xc)))$$  

Conversion of recMoPrP$^{23-231}$ in the presence of Congo red at 1:1, 1:2 and 1:4 PrP to Congo red molar ratios generated the most visually striking results on RENAGE. Figure 3(c,d) shows the result of ShIC-RENAGE using 0.3 mg/mL recMoPrP$^{23-231}$ in the presence of a 1:2 PrP to Congo red molar ratio.
the presence of the PrP monomer persists over three times longer than when the experiment is conducted without Congo red. The presence of small oligomers and large oligomers also persists much longer in the presence of Congo red. Furthermore, the peak intensity of the small oligomer band (Figure 3(d)) is decreased compared to the quantity of small oligomer present in the methanol (control) blank conversion (Figure 3(a)). Comparison of fibril growth kinetics in the presence of Congo red show a delay with a mid-log point of 16 ± 2.8 hours with Congo red compared to 6.9 ± 0.18 hours in the methanol blank (0.46% vol/vol). Furthermore, the rate of fibril growth is decreased substantially with Congo red such that the rate is 0.16 ± 0.03 units/hour versus 0.61 ± 0.05 units per hour in the methanol blank.

**Effect of anti-prion fibril small molecules on PrP ShIC**

The effect of the anti-prion fibril drugs on ShIC of PrP can be monitored by four metrics: 1) changes to the small oligomer population (8-mer to 12-mer), 2) changes to the large oligomer population (>14 mer), 3) the lag phase of fibril formation and 4) the speed of fibril growth. The temporal changes to the PrP oligomers were calculated from the area under the curve of the oligomer (8-mer to 12-mer) population ratio over time. The lag phase was determined from the mid-point of the logarithmic fibril growth and the speed of fibril growth was determined from the slope of the sigmoidal function that was used fit to the fibril growth curve.

The experimental and analytical parameters were developed using PrP ShIC in the presence of Congo red and curcumin, conducted in five replicates and then applied to all remaining drugs or drug candidates. In particular, we converted recMoPrP\textsuperscript{22-231} in the presence of anti-prion drugs at three different molar ratios of PrP-to-drug ranging from 1:1 to 1:4. Next, we determined how GN8, quinacrine, chlorpromazine, TUDCA, and tetracycline, modulate oligomer and fibril formation in ShIC. For these five compounds, ShIC was conducted in triplicate with the drugs at 4-fold and 8-fold the concentration of monomeric PrP, since these drugs were effective at higher concentrations than Congo red and curcumin.

The effect of Congo red, curcumin, GN8, quinacrine, chlorpromazine, TUDCA, and tetracycline on PrP ShIC as measured by the four metrics (small oligomer population, large oligomer, fibril delay and rate of fibril growth) is shown in Figure 4. We found that five of the seven known anti-prion compounds tested, modulated PrP ShIC. The magnitude of the difference between ShIC in the presence of drug and the methanol blank was calculated from pairwise Student’s t-tests and the resulting p-values are shown in Figure 4. For each anti-prion compound, the most significant (lowest p-value) drug-to-PrP ratio is shown in Figure 4. The primary impact of five of the anti-prions, Congo red, curcumin, GN8, quinacrine and chlorpromazine on prion conversion is that they appear to modulate the small oligomer population (Figure 4(a)). Most of the compounds (curcumin, GN8, quinacrine and chlorpromazine) caused an increase in the small oligomer population. In contrast, Congo red caused a decrease in the small oligomer population (at the drug to PrP ratio of 1:1). Significant effects on the large oligomer populations were seen for Congo red, quinacrine and chlorpromazine (Figure 4(b)). The same five anti-prion compounds (Congo red, curcumin, GN8, quinacrine, and chlorpromazine) that affected the PrP oligomer population, also caused a significant delay in the fibril formation. In addition, a significant effect on the slope of fibril growth was only seen for treatments with Congo red and curcumin. Tetracycline caused a less significant (p < 0.1) increase in fibril growth rate. These results suggest that different anti-prion drugs have different mechanisms of action. The drugs that did not have significant impact on RENAGE analysis of prion conversion were TUDCA and tetracycline.

The effects of all the anti-prion molecules analyzed by ShIC RENAGE are summarized in Table 1, along with the comparison to published inhibitory concentration resulting in 50% reduction (IC\textsubscript{50}) of PK resistance from cell-based ScN2A assays. The two most robust and effective measures of anti-prion fibril activity are, firstly, a change in the small oligomer population, and secondly, the delay in fibril growth as measured at the mid-logarithmic point. The lag phase or mid-logarithmic point is a measure of the initiation of fibril formation and thus indicates changes to the fibril nucleation mechanism. This suggests that one of the mechanisms of action of Congo red, curcumin, GN8, quinacrine, and chlorpromazine is to inhibit fibril nucleation. Nucleation inhibition may occur by preventing misfolding through the binding and stabilization of monomeric PrP or by disrupting the fibril seeds. An effect on the fibril growth rate is consistent with drugs that disrupt fibril extension, secondary nucleation or shuttle aggregates into non-fibrillar aggregates. Drugs that affect the fibril growth rate are Congo red and curcumin. Anti-prion molecules that do not appear to have a significant effect via our ShIC-RENAGE assay are tetracycline and TUDCA. Interestingly these two drugs have high IC\textsubscript{50} ScN2A values of 100 µM and in the case of TUDCA, it is only effective in cells after second passage through ScN2A cells [37].
Figure 4. Effect of known anti-prion compounds (Congo red, curcumin, GN8, quinacrine, chlorpromazine, and tetracycline) on oligomer and fibril formation in ShIC of recMoPrP23–231 as measured by the oligomer population (a), large oligomer (b), midpoint of fibril formation (c) and rate of fibril growth (d). The small oligomer and large oligomer population is the area under the time course of these species. The midpoint and rate of fibril growth are determined from the sigmoidal function fit to the time course. Only significant and near-significant results are shown. Bars are labelled with the p-value for 95% significance interval and ‘ns’ indicates not significant.
In order to determine if other compounds of unknown prion activity modify PrP ShIC, we analyzed the effect of DMSO, methanol, thiamine, ferulic acid, gallic acid and melatonin on PrP ShIC. We found that these compounds do not have an effect on the kinetics of PrP conversion by ShIC (results not shown). The full statistical analysis of the lack of effect on ShIC RENAGE by ferulic acid, gallic acid and melatonin are shown in Suppl. Figure 3. These three compounds inhibit in vitro amyloid-β peptide fibril formation [38–40], while the others do not have any known effect on prion or fibril formation. Thus, the effects seen in the ShIC-RENAGE assay are not generic effects arising from anti-amyloid compounds (polyphenols and aromatics).

**Concentration dependent drug effects on PrP ShIC**

Monitoring the effect of different drug concentrations in a drug screening assay is important for identifying the effective concentration that cause significant changes on the ShIC of PrP. For curcumin, the threshold for detecting an effect on the small oligomer population, fibril midlog point and slope of fibril growth is reached at a molar ratio of 1:2 PrP to curcumin. For Congo red, the effect on the small oligomer population and fibril midlog point is seen at a molar ratio of 1:1 PrP to Congo red. Furthermore, for some drugs significant dose dependence is seen during the shaking induced conversion process. For instance, Congo red at molar ratio of PrP-to-drug of 1:1, 1:2 and 1:4 shows a dose dependent decrease in the small oligomer population (Suppl. Figure 4A). In addition, reverse dose dependence is seen in the fibril growth rate for Congo red (Suppl. Figure 4B), with the largest decrease in growth rate seen a 1:1 ratio. This reverse dependence may be due to Congo red inducing formation of different fibrillar aggregates that are in the RENAGE fibril band.

**Identification of novel Anti-Prion Fibril compounds with PrP ShIC and cell-based assays**

After demonstrating that our ShIC-RENAGE assay recapitulates the activity of known anti-prion molecules, we tested ShIC-RENAGE on three new potential anti-prion compounds: rosmarinic acid, myricetin, and resveratrol. We compared these potential anti-prion compounds to ferulic acid, which we determined did not modify ShIC (Suppl. Figure 3). These compounds were identified as potential anti-prion compounds based on their known anti-amyloid activity against Alzheimer’s β-amyloid (Aβ) peptide. Rosmarinic acid, myricetin, and resveratrol are all known to be active in animal models of Alzheimer’s disease [39,41]. In addition, rosmarinic acid, myricetin and resveratrol modulate in vitro Aβ fibril and oligomer formation [42,43]. In contrast, ferulic acid does not affect the pathology in Alzheimer’s mouse model (Tg2576) but does inhibit in vitro amyloid-β peptide fibril formation, extension and destabilize preformed fibrils [38,39]. We were interested in compounds active against Aβ because it has been suggested that certain anti-amyloid drugs will also be anti-prion drugs that act on PrP isoforms [44].

Resveratrol and rosmarinic acid were analyzed in five replicates for three small molecule concentrations of PrP-to-drug molar ratio of 1:2, 1:4 and 1:8. Myricetin was analyzed in triplicate for concentrations at 1:4 and 1:8 molar PrP-to-drug ratio, while the effect of ferulic acid was analyzed at a molar ratio of 1:4. This ShIC-RENAGE analysis revealed that rosmarinic acid and myricetin have activity against PrP aggregation (Figure 5 and Table 2).

The assay results in Tables 1-2 use recombinant recMoPrP223–231 purified from the same batch. Rosmarinic acid modulates the small oligomer and large oligomer population and causes a delay in fibril growth (Figure 5(a,c)). Theses changes are seen at a 1:4 rosmarinic to PrP molar ratio. Interestingly, rosmarinic acid had the largest and most significant effect on the large oligomer population out of all 11 drugs tested in this study. Large-scale changes to the fibril formation, caused by rosmarinic acid at a PrP-to-drug molar ratio of 1:8, was apparent by non-sigmoidal fibril growth for three out of five replicates. Resveratrol did not have a significant effect on any of the measurements from RENAGE analysis of

**Table 1. Effect of anti-prion molecules on shaking induced conversion (ShIC) compared to cell-based ScN2A assay.**

| Anti-prion molecule | Oligomer population (total) | Large oligomer population | Fibril lag (midlog) | Fibril growth rate (slope) | ScN2A assay IC₅₀ |
|---------------------|-----------------------------|---------------------------|---------------------|---------------------------|------------------|
| Congo red           | 0.0008                      | 0.02                      | 0.0007              | 0.001                     | 14 nM            |
| Curcumin            | 0.002                       | ns                        | 0.01                | 0.005                     | 10 nM            |
| GN8                 | 0.04                        | ns                        | 0.01                | ns                        | 1.4 µM           |
| Quinacrine          | 0.03                        | ns 0.06                   | 0.009               | ns                        | 0.4 µM           |
| Chlorpromazine      | 0.01                        | 0.008                     | 0.004               | ns                        | 3 µM             |
| TUDCA               | ns                          | ns                        | ns                  | ns                        | 100 µM           |
| Tetracycline        | ns                          | ns                        | ns 0.1              | ns 0.09                   | 100 µM           |

ns Indicates that result is not significantly different from the control at a 95% confidence interval (p > 0.05)

¹ IC₅₀ after second passage

ns Indicates no activity in ScN2A assay
ShIC-RENAGE. Myricetin was an anti-prion fibril drug by ShIC-RENAGE analysis, by slowing fibril growth and causing a slight delay fibril formation, but with only 90% confidence in the difference between the drug treatment and the control (p = 0.1). However, myricetin caused large scale changes to fibril growth at a PrP-to-drug molar ratio of 1:8, with rapid fibril/aggregate growth following a bidose response curve. Resveratrol and ferulic acid did not have a significant effect on any of the measurements from ShIC-RENAGE analysis. Ferulic acid is shown in Figure 5 for comparison to the other anti-prion drugs.

Table 2. Effect of rosmarinic acid, resveratrol, myricetin, and ferulic acid on ShIC compared to cell-based ScN2A assay.

| Anti-prion molecule | Oligomer population (total) | Oligomer population (large) | Fibril lag (midlog) | Fibril growth rate (slope) | ScN2A assay IC50 |
|---------------------|-----------------------------|----------------------------|---------------------|---------------------------|-----------------|
| Rosmarinic Acid     | 0.02                        | 0.0008                     | ns                  | ns                        | 50 µM           |
| Resveratrol         | ns                          | ns                         | ns                  | ns                        | 100 µM          |
| Myricetin           | ns                          | 0.07                       | ns                  | ns                        | ns              |
| Ferulic Acid        | ns                          | ns                         | ns                  | na                        | ns              |

* Myricetin at 1:4 ratio in ShIC results in sigmoidal fit (p-value shown), but at 1:8 ratio the fibril growth is a bidose fit and kinetics could not be compared.

na indicates no activity in ScN2A assay

Figure 5. Effect of rosmarinic acid, myricetin, and ferulic acid on ShIC of recMoPrP23–231 as measured by the oligomer population (a), large oligomer (b), midpoint of fibril formation (c) and rate of fibril growth (d). Bars are labelled with the p-value for 95% significance interval and 'ns' indicates not significant.

ShIC-RENAGE. Myricetin was an anti-prion fibril drug by ShIC-RENAGE analysis, by slowing fibril growth and causing a slight delay fibril formation, but with only 90% confidence in the difference between the drug treatment and the control (p = 0.1). However, myricetin caused large scale changes to fibril growth at a PrP-to-drug molar ratio of 1:8, with rapid fibril/aggregate growth following a bidose response curve. Resveratrol and ferulic acid did not have a significant effect on any of the measurements from ShIC-RENAGE analysis. Ferulic acid is shown in Figure 5 for comparison to the other anti-prion drugs. Only a molar ratio of 1:4 was used because previous
analysis suggested that the drug did not have anti-prion activity and most drugs that were effective, showed significant modulation at concentrations of PrP-to-drug molar ratio of 1:4.

To confirm the results of our ShIC-RENAGE analysis we tested the effect of resveratrol, rosmarinic acid, myricetin and ferulic acid in a cell-based assay. In this assay we treated scrapie infected neuroblastoma cells (ScN2A) grown to 50% confluency with 50 or 100 µM of each drug for three days. Cells were then lysed and the cell extract digested with proteinase K (+ PK), along with a control without PK (Figure 6(a)). Anti-prion activity was determined based on a decrease of PK resistance without a decrease in overall PrP levels (seen in the - PK control). We found that trans-resveratrol, rosmarinic acid and myricetin have anti-prion activity. Ferulic acid had no effect on the ScN2A cell assay. We also tested the effect of pterostilbene in ScN2A cells because of the structural similarity to resveratrol. Pterostilbene showed a decrease in PK resistance but also caused a decrease in total PrP levels in ScN2A cells. The identification of rosmarinic acid and myricetin as anti-prion molecules confirms our ShIC-RENAGE analysis, which identified these molecules as potential anti-prion fibril drugs. However, ShIC-RENAGE analysis did not identify resveratrol as an anti-prion fibril molecule. This result is consistent with the conclusion that resveratrol does not act directly on PrP isoforms to modulate misfolding, but rather appears to act via other cellular factors.

The inhibitory concentration where there is a 50% reduction in PK resistance (IC₅₀) for rosmarinic acid and myricetin was determined by treating ScN2A cells with 25, 50, 100 and 200 µM (Figure 6(b)). Rosmarinic acid does not alter cell viability up to 250 µM after 24 hrs in N2A cells [45]. Myricetin has a median lethal dose (LD₅₀) of 100 µM in human umbilical vein endothelial cells after 24 hrs incubation [46]. At 20 µM myricetin has no lethal effects after 24 hrs in human neuroblastoma cells [47]. The IC₅₀ based on a plot of the ~ 29 kDa band intensity for rosmarinic acid and myricetin on PrPSc formation was determined to be 49 µM and 44 µM, respectively. There is a decrease in total PrP abundance when cells are treated with 200 µM of both drugs, although the IC₅₀ concentration is 4X lower. Overall, the results seen with the cell-based assay align with the discovery from

Figure 6. Cell-based assay was used to identify anti-amyloid small molecules that have anti-prion activity. Western blot of PrP with and without proteinase K (PK) digestion, from scrapie infected neuroblastoma cells (ScN2A) that were incubated with pterostilbene, trans-resveratrol, rosmarinic acid, myricetin, and ferulic acid, at 100 and 50 µM (panel a). The blank had the same volume of dimethyl sulfoxide (DMSO) added as the drug tests. The effect of four concentrations of rosmarinic acid and myricetin (25, 50, 100 and 200 µM) on PK resistance in ScN2A was used to determine the IC₅₀ (panel b). The gel used to generate the PK digested panels in panel B is shown in Suppl. Figure 6.
ShIC-RENAGE that rosmarinic acid and myricetin are anti-prion compounds.

**Fibrils formed by PrP ShIC in the presence of anti-prion and anti-prion fibril compounds**

We used proteinase K (PK) digestion, SDS-PAGE and negative stain TEM to characterize the end products of shaking induced converted MoPrP^{223–231} in the presence of anti-prion drugs. PK resistance is a hallmark of naturally occurring infectious prions, as well as many recombinant PrP* de novo* converted PrP fibril forms [8, 22]. Changes in the PK resistance due to anti-prion fibril drugs will indicate if the drugs caused large scale disruption of the fibril structure. Fibrils formed in the presence of anti-prion drugs and untreated monomeric recMoPrP^{200–231} were digested with PK at 1:50, 1:200 or 1:400 PrP-to-PK weight ratio. Figure 7 shows the 35 to 11 kDa region of these samples separated on 12% acrylamide SDS-PAGE to reveal PK resistant fragments. Although there are subtle differences in the PK digest patterns from fibrils formed in the presence of the anti-prion or anti-amyloid compounds, the patterns indicate that PK resistant fibrils/aggregates are formed in the presence of all of the drugs we tested. Only myricetin at a ratio of 1:8 PrP-to-drug caused a PK digestion pattern that suggested gross structural changes to the fibril (Figure 7). In contrast, the PK digestion pattern of the end-product formed by ShIC at a lower concentration of myricetin (1:4 PrP to myricetin), was consistent with *de novo* PrP fibrils (result not shown). We performed the PK digest in triplicate and quantitatively compared the ~18 kDa band to the methanol control. These results showed only a significant (p < 0.05) decrease in the ~18 kDa band for curcumin and myricetin (results not shown). In addition to the PK digestion pattern, SDS-PAGE analysis also revealed that the fibrils formed in the presence of myricetin (1:4 and 1:8) and rosmarinic acid (1:4 PrP-to-drug) are resistant to boiling in SDS loading buffer (Suppl. Figure 5).

Negative stain TEM can be used to reveal if anti-prion molecules influence the gross PrP fibril morphology or indicate aggregation to off-pathway products. Fibrils formed by ShIC in the presence of all the small molecule drugs in this study were analyzed by negative stain TEM (Figure 8). As seen previously, ShIC of recMoPrP^{223–231} formed rod-like fibrils [25]. Conversion in the presence of the anti-prion compounds, except for myricetin, also formed small rod-like fibrils. Converting recMoPrP^{223–231} in the presence of myricetin at 1:8 PrP-to-drug molar ratio led to only amorphous aggregates and no fibrils.

**Discussion**

Our results reveal the effect of 10 anti-prion as well as 6 prion-inactive or anti-amyloid compounds on *de novo* recombinant PrP conversion, under non-denaturing conditions at near physiological pH. For the 6 prion-inactive compounds tested, the false positive rate in detecting an anti-prion compound effect on PrP ShIC was 0%. In our analysis of 10 anti-prion compounds, ShIC RENAGE analysis revealed a detectable effect on 8 out of the 10 anti-prion compounds tested. Therefore, ShIC RENAGE has a false negative rate for detecting anti-prion compounds of 20%. The 10 anti-prion compounds were selected because they were either: 1) known to disrupt prion formation by directly interacting with PrP isoforms, or 2) known to have anti-amyloid activity. We identified, rosmarinic acid and myricetin as new anti-prion fibril molecules using the kinetic and endpoint measures of our ShIC-RENAGE assay. Furthermore, we demonstrated that rosmarinic acid and myricetin are anti-prion compounds in a cell-based assay. Rosmarinic acid and myricetin are polyphenols that are present in lemon balm, rosemary, oregano, sage, thyme and peppermint. Myricetin is abundant in grapes, berries and wine. However, trans-resveratrol was not identified as an anti-prion fibril drug via ShIC-RENAGE even though it had anti-prion activity in the cell-based assay. This was likely due to the fact that trans-resveratrol mediates its anti-prion activity through other cellular factors (and not PrP). In fact, studies on the action of anti-prion compounds show that most anti-prion compounds identified in cell-based screens do not bind to or modify PrP isoforms [48]. Therefore the PrP ShIC-RENAGE assay is inherently prone to result in false negative characterization of anti-prion compounds.

This is the first time that the effect of rosmarinic acid, myricetin, GN8, quinacrine, and chlorpromazine on PrP conversion have been monitored by time-dependent analysis. Furthermore, the ShIC-RENAGE method appears to be the first method that allows anti-prion time-dependent activity on PrP oligomers to be monitored and characterized. Previously real-time analysis has been done for the effect of Congo red, curcumin, tetracycline, and TUDCA in guanidinium-induced conversion, as monitored by thioflavin T fluorescence [37, 49]. Also, the effect of TUDCA on RT-QuIC converted prions is now available [37]. Previous attempts to perform time-dependent analysis of quinacrine activity on prion conversion failed because the required concentrations of quinacrine interfere with Thioflavin T fluorescence detection [50]. Notably, previous cell-free full-length PrP conversion methods for
identifying and characterizing anti-prion molecules have relied on denaturants or detergents that often disrupt ligand to protein interactions [22, 24, 51, 52]. Even RT-QuIC often contains low concentrations of SDS (~ 0.05%) [51, 52].

**Identifying mechanisms of anti-prion effects**

Conversion of full-length PrP by the ShIC method can be used to identify effective small compounds that act directly on PrP targets to inhibit PrP oligomerization and fibrilization. Our ShIC-RENAGE assay identified drugs that separately modulate oligomer, seeding and fibril formation. Drugs that stabilize or destabilize PrP oligomers are likely to also modify the nucleation or seeding capability. In fact, all of the anti-prion fibril molecules that we identified as modifying the small oligomer population also caused a delay in the initiation of fibril formation (midlog) (Tables 1-2). Modification of the oligomer population may occur due to drugs that interact with PrP[C] or the oligomers.

The role of oligomers in synthetic PrP fibrillization and similarly pathogenic prion formation is complex. Oligomers (~ 20 monomers) isolated from

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**Figure 7.** PK digestion of recMoPrP[23−231] compared to fibrils revealed that fibrils are formed by ShIC of PrP in the presence of anti-prion molecules. SDS-PAGE (12% acrylamide) of (a) recMoPrP and fibrils formed by ShIC in the presence of (b) 0.046% methanol blank (c) 1:2 PrP-to-Congo red, (d) 1:2 PrP-to-curcumin, (e) 1:8 PrP-to-GNB, (f) 1:4 PrP-to-quinacrine, (g) 1:4 PrP-to-chlorpromazine, (h) 1:8 PrP-to-TUDCA, (i) 1:8 PrP-to-tetracycline, (j) 1:4 PrP-to-rosmarinic acid, (k) 1:4 PrP-to-resveratrol, (l) 1:8 PrP-to-myricetin, and (m) 1:4 PrP-to-ferulic acid.
prion-infected cells are highly infectious [53]. PrP oligomers and fibrils that constitute so-called synthetic prions are not infectious with the high titres characteristic of pathogenic PrP<sup>Sc</sup> [54]. Therefore, we expect structural differences between oligomers and fibrils formed by ShIC and oligomers found in prion-infected cells. Our result showing that some anti-prion compounds increase the oligomer population while other compounds decrease the oligomer population may be due to a heterogeneous mix of oligomers being formed. Some oligomers involved in prion fibril formation are more infectious than others [53,55]. In our de novo formation of oligomers by ShIC, the mechanism by which anti-prion molecules increase the number of oligomers formed is likely due to stabilizing off-pathway or less toxic oligomers that may have the same size but are structurally and kinetically different. We propose that ShIC identifies compounds that act on the inherent protein oligomerization and fibrillation properties of PrP, similar to anti-amyloid compounds. Our results are consistent with anti-amyloid activity being one of the mechanisms of anti-prion activity.

Our result showing that anti-prion fibril compounds delay nucleation is significant for their use as potential anti-prion prophylactics. The nucleation of a PrP<sup>Sc</sup> seed is a critical step in genetic and spontaneous prion disease. In humans only 1% of prion disease is acquired [56]. Remarkably, a mouse model of spontaneous prion disease is available that expresses a mouse PrP mutant (170N 174T) with the elk PrP rigid loop and results in transmissible and pathogenic prions [57]. Discovering anti-prion compounds with prophylactic activity in preventing or delaying prion formation due to genetic disease, is of high value to improving health outcomes in patients diagnosed with a genetic predisposition for prion disease.

**Congo red** is known to directly bind to, and modify, PrP isoforms and plaques in a strain-dependent manner [31,58]. Furthermore, other cell-free analyses indicate that Congo red binds PrP<sup>C</sup> non-specifically, inducing aggregation and inhibiting new fibril formation [24,59]. ShIC-RENAGE analysis show that Congo red effectively decreased the PrP oligomer population, delayed the fibril lag phase and slowed fibril growth. In guanidinium-induced conversion, Congo red deceases
the fibril yield but the lag phase is only slightly affected while the growth rate is not affected [49]. Together, these results are consistent with a mechanism whereby Congo red interacts with PrP, oligomers and fibrils to decrease primary nucleation, secondary nucleation and prion formation.

**Curcumin** also modifies PrP isoforms in cells and binds β-rich PrP oligomers and fibrils [32,35]. ShIC-RENAGE analysis show that curcumin effectively increased the oligomer population, delayed initiation of fibril formation and slowed fibril growth. The increase in the oligomer population is consistent with curcumin binding to PrP oligomers. In comparison, curcumin affects guanidinium-induced conversion by decreasing the fibril yield and decreasing the growth rate, but the lag time is unchanged [49].

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**Figure 8.** Negative-stain electron micrographs of fibrils formed by prion ShIC in the presence of (a) 0.046% methanol blank (b) 1:2 PrP-to-Congo red, (c) 1:2 PrP-to-curcumin, (d) 1:8 PrP-to-GN8, (e) 1:4 PrP-to-quinacrine, (f) 1:4 PrP-to-chlorpromazine, (g) 1:8 PrP-to-TUDCA, (h) 1:8 PrP-to-tetracycline, (i) 1:4 PrP-to-rosmarinic acid, (j) 1:4 PrP-to-resveratrol, (k) 1:8 PrP-to-myricetin, and (l) 1:4 PrP-to-ferulic acid. The scale bars = 200 nm. In the presence of most anti-prion molecules fibrillar rods are formed, except in the presence of myricetin.
Quinacrine, Chloropromazine and GN8 bind specifically to PrP\(^C\) as seen by X-ray crystallography [60] and by interacting residues identified by solution NMR [61]. In our ShIC-RENAGE studies of PrP, we found that GN8, quinacrine and chlorpromazine increased the population of small oligomers and delayed fibril formation. Interestingly the effects of quinacrine and chlorpromazine (tricyclic) on ShIC are similar, with quinacrine exerting an effect at a lower concentration (2-fold molar excess) compared to the 4-fold excess required for chlorpromazine. These results are consistent with a mechanism wherein the drug binds and stabilizes PrP\(^C\), stabilizes the population of oligomers and inhibits nucleation. The increase in oligomer population is also consistent with these drugs stabilizing an off-pathway oligomer.

The effects of TUDCA on PrP fibril formation kinetics using guanidinium-induced fibrilization, indicate that it caused a delay in fibrilization and in RT-QuIC TUDCA blocked seeding [37]. In our ShIC-RENAGE analysis, TUDCA had no anti-prion effect. This suggests that TUDCA does not modulate the primary nucleation or prion propagation. What ShIC-RENAGE analysis may not reveal is the secondary propagation events that are consistent with effects of TUDCA seen in the second passage of a ScN2A assay, but not the first passage.

The effectiveness of tetracycline in our ShIC-RENAGE assay on PrP conversion is less significant than with other drugs/compounds. However, the results show that it delays and decreases growth of fibrils. This is consistent with the mechanism revealed in a hamster model of prion disease (263K prion strain) that shows that tetracycline interacts with PrP\(^\text{PrP}^\text{Sc}\) [34].

We identified rosmarinic acid and myricetin as anti-prion fibril molecules in our ShIC-RENAGE assay and confirmed these results in a scrapie cell-based assay. In ShIC-RENAGE assays, rosmarinic acid caused an increase in the small and large PrP oligomer population. In fact, rosmarinic acid increased the population of the large oligomer more than any other anti-prion molecule that we tested. In addition, rosmarinic acid caused a significant delay in fibril formation. On the other hand, our ShIC-RENAGE data shows that myricetin has a more modest effect, as it only decreased the rate of fibril growth. Both rosmarinic acid and myricetin change the fibril end product as seen by SDS-resistance. Furthermore, myricetin changes the PK digestion pattern and fibril morphology as seen by TEM. Based on these results we propose a mechanism whereby rosmarinic acid acts by stabilizing PrP oligomer formation, inhibiting seeding and the production of an off-pathway prion fibril. Myricetin induces the formation of off-pathway aggregates, with the formation of non-fibrillar aggregates as seen in our TEM studies.

**Comparison to cell-based assays and animal models**

The anti-prion small molecules chosen for this study were well characterized and their inhibitory effects on reducing the amount of PK-resistant PrP isoform (PrP\(^\text{PrP}^\text{res}\)) in ScN2A were known. In addition to the seven known anti-prion compounds, we identified trans-resveratrol, rosmarinic acid and myricetin as three new anti-prion molecules. Congo red and curcumin have nanomolar inhibitory concentrations, at which there is a 50% decrease of PK resistant PrP fibrils or PrP\(^\text{PrP}^\text{Sc}\) (IC\(_{50}\)), of 14 and 10 nM, respectively [31,32]. Despite Congo red being unable to cross the blood-brain barrier [58], it has been shown to increase survival by 8–10% in a hamster model of prion disease [36]. The effectiveness of curcumin in mouse models is limited with no significant effect in one study [32] and an 8% increase in survival when at a low dose but no effect at a higher dose in another study [32,62]. Quinacrine has a sub-micromolar IC\(_{50}\) of 0.4 \(\mu\)M. Despite its use in human clinical trials and two separate mouse scrapie challenge studies, quinacrine has no demonstrated *in vivo*, anti-prion effect [30,63].

**Role of ShIC-RENAGE in anti-prion discovery**

We believe that anti-prion molecules that affect PrP oligomer and fibril formation may have the most promise as potential drug leads. This is because the highly effective arylhydrazone (so called compound B), anle138b and cellulose esters are all known to act directly on PrP isoforms, to modify PrP oligomers, using guanidinium-induced fibrilization, indicate that it caused a delay in fibrilization and in RT-QuIC TUDCA blocked seeding [37]. In our ShIC-RENAGE analysis, TUDCA had no anti-prion effect. This suggests that TUDCA does not modulate the primary nucleation or prion propagation. What ShIC-RENAGE analysis may not reveal is the secondary propagation events that are consistent with effects of TUDCA seen in the second passage of a ScN2A assay, but not the first passage.

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Materials and methods

Denaturant-induced conversion of recprp

To assess the efficacy of different PrP conversion methods on different prion constructs we generated oligomers from recMoPrP90–231 (recombinant mouse prions from residue 90 to 231) and recShPrP90–232 (recombinant Syrian hamster prions from residue 90 to 232) using more conventional prion conversion conditions: 3 M urea, 20 mM sodium acetate, pH 4 and 200 mM NaCl [66], acid induced conversion at pH 1 for 2 hours [67], and LPS induced conversion [29].

Cell-based assay

Scrapie-infected neuroblastoma N2A cells (ScN2A) were grown in Opti-MEM with 10% FBS to ~ 50% confluency prior to the addition of test compound, after which cells were incubated at 37 °C for 3 days. Cells were then lysed with lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 10 mM Tris-HCl, pH 7.5) for 10 minutes at 4 °C. Cell debris were removed by centrifugation and protein concentration of the supernatant was determined. Proteinase K analysis was performed by treating 50 μg of each sample with or without 50 μg/mL PK for 1 hour at 37 °C. Samples were analyzed by 4–12% gradient SDS-PAGE, western blotted and probed with 6D11-biotin and streptavidin-HRP. Blots were developed with ECL kit (BioRad).

Recprp purification

Full-length recombinant mouse prion protein and truncated mouse or Syrian hamster PrP (recMoPrP23–231, recMoPrP90–231, recShPrP90–232) with His6x-tags were expressed in E. coli and purified as previously described [25,26,67]. Briefly, all constructs were purified on NiNTA (Qiagen Canada, Toronto, Canada) and a Peirce Protease Inhibitor (Thermo Fisher) was added to eluted PrP fractions at a dilution of between 100X and 25X, from a mini-tablet dissolved in 1 mL of water. In addition, for the full-length recMoPrP23–231, 1 mM EDTA was added to the eluted PrP fractions. Purified protein fractions were then dialyzed first into 20 mM sodium acetate pH 5.5, followed by 18Ω water and then lyophilized. The purity of all constructs was confirmed to be greater than 98% by SDS-PAGE. The protein concentrations were determined using absorption extinction coefficients determined by the ProtParam webserver (ExPASy).

Resolution enhanced native acidic gel electrophoresis (RENAGE)

The relative quantity of the prion monomers, oligomers and fibrils in converted samples were analyzed using a specially developed technique called RENAGE [26]. Gels were prepared using a 8% acrylamide pH 4.3 running gel and a 3% acrylamide pH 5.2 stacking gel, as previously described [26]. The running buffer consisted of 0.35 M β-alanine and 0.14 M acetic acid at pH 4.3. Samples were mixed with 5X dissolving buffer (37% glycerol, 128 mM acetic acid-KOH, pH 5.2 and 0.01% crystal violet (Sigma-Aldrich Canada)). Gels were pre-run at 30 mAmp per gel at normal polarity for 17 minutes and then 5 μg of the prion samples were loaded in dissolving buffer. The gels were run at 30 mAmp with reverse polarity for ~ 85 minutes for truncated recombinant PrP samples and 90 minutes for full-length recMoPrP23–231 samples. Gels were stained with colloidal Coomassie blue for at least four hours and destained in water [68]. The size of the truncated PrP oligomers was determined using a PrP PICUP ladder that was generated by photo-induced cross-linking of unmodified protein (PICUP) of recMoPrP90–231 at 1 mg/mL as previously described [26]. The size of recMoPrP23–231 oligomers was determined previously [26], using a ladder of bovine serum albumin (BSA) oligomers and the estimated mass to charge ratio of BSA.

High-throughput RENAGE

RENAGE analysis of 120 ShIC time-course sets (1440 samples) required a high throughput electrophoresis method. For this purpose, twelve RENAGE gels were poured at once using a Mini-PROTEAN 3 multi-casting chamber. After pouring the running gel, water-saturated butanol was added on top (0.2 mL per gel), immediately followed by layering on 0.5 mL water, before the gel set. The water layered onto the water-saturated butanol was required for the stacking gel to adhere to the running gel. The gels were allowed to polymerize at 22 °C to 25 °C [26]. Twelve gels were run at the same time on a mini-PROTEAN 3 Dodeca cell. Each gel consisted of all time points for each ShIC sample set. In addition, a standard consisting of 2 μg recMoPrP90–231 oligomers and 2 μg recMoPrP23–231 was loaded on each gel. This standard was used to correct differential staining of oligomers versus monomeric PrP in different gels. Gels were stained as described above.
*Shaking-induced conversion (ShIC) of recPrP to oligomers and fibrils*

Recombinant PrP was converted to β-sheet rich oligomers and fibrils using shaking alone in 1.5 mL tubes, as described previously [25]. Initial testing of the effects of known anti-prion molecules on ShIC was performed by adding the anti-prion molecule from 5 mM stocks in DMSO to 0.5 mg/mL recShPrP[20–232] at a molar ratio of 1:1 PrP to anti-prion compound. The effect of the anti-prion compounds was monitored by RENAGE. Time-dependent monitoring of ShIC of PrP was developed using full-length recMoPrP[23–231] so that conversion of recPrP to fibrils was complete by 24 hours. Briefly, standard conditions for conversion of all PrP constructs used 0.4 mL of a 0.5 mg/mL recMoPrP[23–231] solution in 20 mM sodium acetate, pH 5.5, that was shaken at −300 rpm in a Lab-line 3529 orbital shaker (orbit diameter of 2.5 cm), at 37 °C in a 1.5 mL polypropylene MaxyClear microcentrifuge tube (Corning Life Sciences − Axygen, Union City, USA). The tube was taped horizontally onto an orbital platform for shaking.

**ShIC in a 96-well plate format**

Testing the effect of known anti-prion compounds on ShIC of PrP was performed on a semi-high throughput scale in a 96-well Deep Well polypropylene plate (ThermoFisher, No.: 260,251), sealed with Well Cap (Natural) (Thermo Fisher, No.: 276,002). The concentration of recMoPrP[23–231] was decreased to 0.3 mg/mL as an optimum concentration for loading 5 μg of PrP in one lane of a RENAGE gel. We also compared the effect of the carrier solvents, DMSO and methanol, which were used to solubilize the anti-prion compounds. We found that methanol had less of an effect on the conversion kinetic than DMSO. Anti-prion compounds were added to the wells from 5 or 10 mM stocks, dissolved in methanol to generate samples mixed with a PrP-to-drug molar ratio of 1:1, 1:2, 1:4, 1:8. The anti-prion compound stocks were prepared ahead of time and stored in glass vials at −80°C. The drugs were added to the 96-well plates first and the plate was then sealed and placed at −20°C, until right before adding the PrP mixture. The PrP mixture consisted of 0.3 mg/mL (11.5 μM) recMoPrP[23–231] in 20 mM sodium acetate pH 5.5. This was added to the wells containing drugs and a zero time-point taken. The ShIC anti-prion assay was started by placing the 96-well plate on its side, taped into a flask clamp and shaken at 280 rpm at 37 °C in a S1 600 Jeio Tech orbital shaker (orbit diameter is 30 mm). Samples were taken at 2, 5, 8, 11, 24, 30 and 48 hrs. A duplicate plate was started at the end of the day and time-points taken at 0, 12/13, 15/16, 18/19, 21 and 36 hrs. To take time points the 96-well plates were removed from the shaker, centrifuged for 10 seconds at 1000 rpm in an Allegra X-22 centrifuge, and 15 μL of the sample transferred to a 96-well low protein binding, skirted PCR plate (Fisher Scientific) and stored at 4 °C for RENAGE analysis at the completion of the time course.

Quantitation of monomers, oligomers and fibrils was performed by first scanning the gel image and then converting the gel lanes to a band-intensity chromatogram using ImageJ (http://rsbweb.nih.gov/ij/index.html). Chromatograms were then plotted using the Origin software package (OriginLab Corp., version 9) and the peaks manually marked and integrated, using Origin’s ‘peak analyzer’ module. Percentages of small (7-mer to 12-mer) and large (>14-mer) oligomers were then calculated from the area of each peak compared to the total integrated area. The RENAGE fibril peak areas were plotted versus time and fitted to a sigmoidal function (eq. 1) using the Origin software package.

**Negative stain transmission electron microscopy**

Prion samples converted in the presence of anti-prion compounds were applied to UV irradiated 300 mesh copper grids with a support film of Formvar with carbon (Ted Pella, Inc., Cat# 01753-F, Redding, CA, USA). The samples were diluted to 2 μM PrP in water and 10 μL of the prion solution was spotted onto Parafilm. The grid was placed on top of the droplet for 1 minute. Grids were washed once with 10 μL water and stained twice with 10 μL of 4% uranyl acetate, whereby each time the grid was placed on top of the droplet, the residual solution was wicked away using filter paper. Micrographs were acquired on a Philips/FEI (Morgagni) transmission electron microscope at 80 kV.

**Proteinase K digestion**

The susceptibility/resistance to PK digestion of the fibrils/end products of ShIC in the presence of anti-prion compounds was tested using a method adapted from Atarashi et al. [51]. For digestion, samples at 0.3 mg/mL PrP in 20 mM sodium acetate pH 5.5 were diluted to 0.25 mg/mL and 100 mM Tris pH 8 was added. Then proteinase K (Promega, Madison, WI, USA) was added at weight ratios of 1:400, 1:200 and 1:50, PK-to-PrP. The sample was digested for 45 minutes at 37 °C. After digest, 0.1 mM PMSF was added and the samples were placed on ice. The Laemmli sample buffer (2% SDS, 5% glycerol, 2 mM DTT, 50 mM Tris, pH 6.8, 0.01% bromophenol blue) was then added followed by
2 M urea, which was added from a 10 M stock. These samples were boiled at 95 °C for 5 minutes and loaded onto a 12% SDS-PAGE gel with a Tris-Glycine buffer system. The gel was run at 180 V for 45 minutes and visualized with colloidal Coomassie blue.

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Disclosure statement

There are no potential conflicts of interest or financial interests to disclose.

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